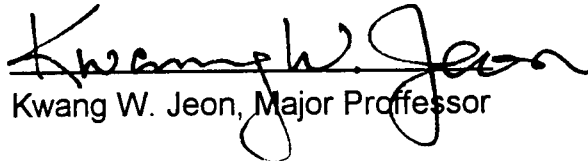
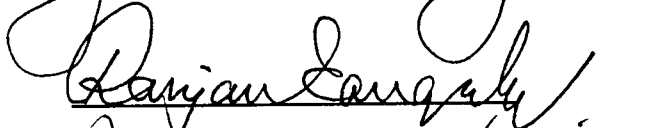
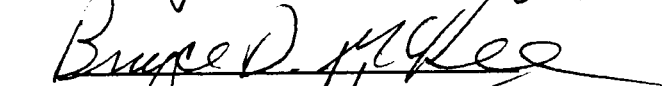


To the Graduate Council:

I am submitting herewith a dissertation written by Sang Wook Oh entitled "Molecular Studies on a Mip-like Protein Required in X-Bacteria/ Amoeba Symbiosis and Characterization of the Myosin Heavy chain Gene in *Amoeba proteus*." 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.


Kwang W. Jeon, Major Professor

We have read this dissertation
and recommend its acceptance:


Accepted for the Council:


Associate vice Chancellor
and Dean of the Graduate School

**Molecular Studies on a Mip-like Protein Required
in X-Bacteria/Amoeba Symbiosis
and
Characterization of the Myosin Heavy Chain Gene
in *Amoeba proteus***

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

**Sang Wook Oh
August, 1998**

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ABSTRACT

X-Bacteria, obligatory endosymbionts in the xD strain of *Amoeba proteus*, survive inside hosts and the symbiosomal membrane containing X-bacteria does not fuse with lysosomes, suggesting that certain component(s) from X-bacteria may contribute to this process. Meanwhile, the macrophage infectivity potentiator (Mip) as a surface polycationic protein is known to be a virulence factor, involved in the initial infection of *Legionella* as well as many pathogenic bacteria. Since recent studies on cell wall components in X-bacteria and nucleotide sequence of the *groELx* gene show that X-bacteria are closely related to *Legionella* species, the *mip*-like gene was cloned and the function of Mip-like protein was investigated in X-bacteria/amoeba interaction.

A 30-kDa Mip-like protein (Mipx) in X-bacteria was detected by SDS-PAGE, 2-dimensional gel electrophoresis and immunoblotting using an antibody against Mip of *Legionella pneumophila*. Immunoblotting of X-bacterial proteins fractionated by detergents showed that Mipx is a membrane protein. Using conserved nucleotide sequences from *Legionella mip* genes as primers, a PCR product containing a *mip*-like gene (*mipx*) was obtained from a genomic expression library of X-bacteria. The presence of *mipx* in X-bacterial genomic DNA was confirmed by Southern hybridization using a ³²P-labeled PCR fragment as a probe.

The *mipx* gene revealed that it contained one open reading frame (ORF) of 729 bp encoding Mipx protein of 243 amino acids. The Mipx protein was highly basic with a predicted pI value of 10.02 and its secondary structure corresponded very well to those of FKBP homologs. The *mipx* gene had a sequence identity of 79%, 74% and 61% with those of *Legionella micdadei*, *L. pneumophila* and *Chlamydia trachomatis*, respectively. Mipx contained amino

acids corresponding to the peptidyl-prolyl *cis-trans* isomerase (PPIase) activity region at its C-terminus and six amino acids out of ten known to be involved in the interaction of human FKBP and FK506 were well conserved in Mipx.

The recombinant Mipx protein overproduced by *E. coli* exhibited PPIase enzymatic activity and its activity was inhibited by the immunosuppressant drug, FK506. For evaluating the role of Mipx in X-bacteria infection in amoebae, X-bacteria pretreated with FK506 were experimentally introduced into D amoebae. X-Bacteria's infectivity in amoebae was lowered to one-third that of the control group, and the results indicated that Mipx may be involved in the initial infection of X-bacteria in amoebae. Since the recombinant Mipx-producing *E. coli* were being almost digested 3 days after infection into amoebae chased by mAb KJX5, the other factors as well as Mipx protein are thought to be involved in the survival of X-bacteria in amoebae.

Monoclonal antibodies against the myosin heavy chain of *Amoeba proteus* were obtained and used to localize myosin inside amoebae and to clone cDNA gene encoding myosin. Myosin was found throughout the amoeba cytoplasm but was more concentrated in the ectoplasmic regions as determined by indirect immunofluorescence microscopy. In symbiont-bearing xD amoebae, myosin was also found on the symbiosome membranes, indicating that myosin is involved in the cellular organelle movement. The localization of myosin in xD amoebae on the symbiosome membrane was further confirmed by immunoelectron microscopy. The ORF of a cloned myosin cDNA gene contained 6,414 nucleotides, coding for a polypeptide of 2,138 amino acids. While amino-acid sequence of the globular head region of amoeba myosin had a high degree of homology with those of myosin from various organisms, the tail region building an α -helical coiled-coil structure did not show a significant sequence similarity. Interestingly enough, the proline residue which is strictly conserved in all

myosins and used as a landmark for the boundary between the head and tail domains was replaced with lysine in the amoeba myosin. There appeared to be at least three different isoforms of myosins in amoebae, with a high degree of homology in their globular head regions.

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CHAPTER I

INTRODUCTION

A. Background of Amoeba-Bacteria Symbiosis

Among host-symbiont relationships, the most fascinating model system is that of amoeba-bacteria not only because new symbiosis can be easily established but also changes of cellular character, including a host's dependence on symbionts, can be experimentally induced under laboratory conditions.

The amoeba-bacteria symbiotic relationship began in 1966 when the D strain of *Amoeba proteus* was spontaneously infected with rod-shaped Gram-negative bacteria (Jeon and Lorch, 1967). The infecting bacteria were called 'X-bacteria' because their origin and identity were not known (Jeon and Lorch, 1967). Hence, the bacteria-bearing amoebae have been known as 'xD amoebae' since. Initially, infecting bacteria were virulent enough to kill almost every culture of amoebae. Some of the amoebae, however, survived. Gradually, the infecting bacteria became less harmful to the host and were eventually transformed from virulent pathogen to essential cell components, so that the host amoebae became dependent on bacterial symbionts for their survival (Jeon, 1972, 1987). Bacterial endosymbionts are sequestered in membrane-bounded vesicles called symbiosomes (Jeon, 1983; Roth et al., 1988) which occupy approximately 10% of xD amoeba's cytoplasm. In fact, each xD amoeba harbors about 42,000 bacterial endosymbionts (Ahn and Jeon, 1979).

With the establishment of a symbiotic relationship, many characteristics demonstrating a mutual dependence for survival developed between xD amoebae

and X-bacteria (Jeon, 1972). First, X-bacteria grew only inside host amoebae and not *in vitro*, suggesting that X-bacteria had become obligatory symbionts of amoebae. Secondly, in high-temperature cultures (26° C), X-bacteria completely disappeared by day 8 and a sharp decrease in the amoeba population followed (Jeon and Ahn, 1978). In addition, the use of prokaryote-specific antibiotics, such as chloramphenicol (Jeon and Hah, 1977), caused the same effects as prolonged high-temperature culture, eventually leading to the death of all host amoebae. Thirdly, the infection of X-bacteria into amoebae caused deletion of the host's S-adenosylmethionine synthetase (SAMS) gene which was compensated for by the presence of symbiotic X-bacteria (Choi et al., 1997). Since S-adenosylmethionine is known to be a principal methyl donor in nucleotide and protein metabolism, the *sams*⁻ mutant cell would not be able to survive without supplemented SAMS enzyme. xD Amoebae could be supplied with SAMS or alternatives only from symbionts, indicating how symbiotic bacteria became essential cellular components. Based on accumulated information, therefore, the relationship between xD amoebae and X-bacteria is considered to be an obligatory symbiosis (Jeon, 1983).

When X-bacteria were allowed to infect amoebae by phagocytosis, they were first found in phagosomes. Most of the X-bacteria were later digested following phagosomal fusion with host lysosomes. Some X-bacteria, however, avoided digestion and became segregated in separate symbiosomes (Ahn and Jeon, 1979). Symbiosomes containing X-bacteria do not fuse with lysosomes and X-bacteria reach maximum numbers within three weeks (Ahn and Jeon, 1979). The failure of symbiosomal fusion with lysosomes gives rise to speculation that the symbiosomal membrane has been modified during its formation and maturation so that it contains some components which actively block lysosomal fusion (Jeon, 1992), particularly since, like phagosomes, the symbiosomal membrane is derived from the host

plasma membrane. Hence, X-bacteria appear to have developed two strategies insuring survival from their initial entry to successful multiplication within the host: resistance to lysosomal enzymes and modification of symbiosome membranes to prevent symbiosome-lysosome fusion.

Isolated X-bacteria were resistant to lysing agents including lysozyme and mild detergents (Han and Jeon, 1980). Further, X-bacteria pretreated with ethidium bromide or acridine orange for curing two plasmids, 21 and 59 kb, failed to infect *D. amoebae* (Han and Jeon, 1980) and were actually digested by amoebae. Thus, it is suggested that plasmid DNAs encode bacterial surface component(s) providing extra resistance to X-bacteria and they may be needed at least for bacterial infectivity.

It is common that symbiosomal membrane component(s) receive attention as 'fusion-blocking' factor(s) in unraveling the mechanisms of symbiosomes-lysosome fusion avoidance. Two symbiosomal membrane components produced by X-bacteria have been found and are suspected to be involved in the prevention of lysosomal fusion.

The presence of a 96-kDa protein on the symbiosomal membrane was determined by indirect immunofluorescence and immunoelectron microscopy (Ahn et al., 1990). The protein was also present in symbiotic bacteria but not in symbiont-free amoebae, suggesting that it was produced by X-bacteria and played a role as a 'fusion-blocking' factor. Several monoclonal antibodies (mAb) against the lipopolysaccharide (LPS) of X-bacteria recognized the antigens not only on the X-bacteria cell surface but also on the symbiosomal membrane. When an anti-LPS mAb was microinjected into *xD* amoebae, the symbiosomal membrane was specifically stained (Choi and Jeon, 1992), showing that X-bacterial LPS were exposed on the cytoplasmic side of symbiosomal membranes. In addition, double-staining experiment using anti-LPS mAb and anti-lysosomal-membrane protein mAb

showed that the coating of the symbiosomal membrane with an anti-LPS mAb by microinjection permitted lysosomal fusion with symbiosome (Kim et al., 1994). Thus, LPS appeared to be involved in preventing symbiosome-lysosome fusion through an unknown mechanism involving interactions with the lysosomal membrane.

While the symbiosomal membrane components were studied, several host-derived cytoskeletal proteins have been detected on symbiosome membranes as well as symbionts. Spectrin subtypes of 225 and 220-kDa were present on symbiosomal membranes (Choi and Jeon, 1989a, b) and 43-kDa amoeba actin was selectively accumulated in symbiosomes and attached to the surface of X-bacteria (Kim and Jeon, 1987). Further, a 210-kDa myosin was recently detected on symbiosomal membranes by immunoelectron and immunofluorescence microscopy (Oh and Jeon, 1998). Whereas the roles of these cytoskeletal proteins on symbiosomes are not known, they seem to be involved in the formation and maintenance of the symbiosome (Jeon, 1997).

B. Mip Protein

The macrophage infectivity potentiator (Mip) protein, a polypeptide of 24 kDa, was first identified and sequenced from *Legionella pneumophila* (Engleberg et al., 1984; Engleberg et al., 1989). This protein has been shown to be present on the surface of bacteria and is highly basic (pI 9.8). Even though Mip seemed at first to be unique to *L. pneumophila*, a number of Mip-like proteins, all 24 - 30 kDa, have since been found in other species of *Legionella* (Cianciotto et al., 1990a), *Chlamydia trachomatis*, an obligate intracellular bacterium (Lundemose et al., 1991), some species of *Rickettsiaceae* including *Coxiella burnetti* (Dumais-Pope et al., 1993), the

intracellular protozoan parasite *Trypanozoma cruzi* (Moro et al., 1995), *Escherichia coli*, and other species of enterobacteriaceae (Horne and Young, 1995).

The Mip protein family from the genus *Legionella* was studied in detail. *Legionella*, pathogenic facultative intracellular Gram-negative bacteria, are able to infect human monocytes and alveolar macrophages (Horwitz and Silverstein, 1980; Nash et al., 1984), thus causing severe cases of pneumonia. The microorganism also survives in cultured Chinese Hamster Ovary cells (McCusker et al., 1991), macrophage-like cultured cell lines such as U937 and HL60 cells (Pearlman et al., 1988; Marra et al., 1990), and in protozoa including a variety of amoebae and *Tetrahymena* (Rowbotham, 1986). Once phagocytosed, *Legionella* are able to reduce the oxidative burst, and inhibit phagosome-lysosome fusion and acidification of the phagolysosomal compartment (Horwitz, 1988).

The DNA sequence of the *mip* gene was first determined from *L. pneumophila* Philadelphia strain 1 followed by five *L. pneumophila* isolates (Ludwig et al., 1994) and one strain of the pathogenic species *L. micdadei* (Bangsberg et al., 1991). While deduced amino acid sequences of *mip* showed 71% homology between the *L. pneumophila* and *L. micdadei*, *L. pneumophila* Mip proteins from different strains showed only a single amino acid difference at position 135. The Mip protein appears to be required for full virulence and intracellular survival of *Legionella* since null mutants of *mip* have reduced virulence and survivability in guinea pigs and cultured macrophages (Cianciotto et al., 1989, 1990b). It seems that such mutants are defective in the initial infection into host cells. Using allelic exchange mutagenesis, *L. pneumophila* and *L. micdadei* mutants that completely and specifically lacked a 24-kDa Mip protein, were constructed (Cianciotto et al., 1989; O'connell et al., 1995). Since the *mip*⁻ mutant strain exhibited an 80-fold loss of infectivity for both U937 cells and human alveolar macrophages *in vitro* (Cianciotto et al., 1990) but regained full

infectivity upon reintroduction of a cloned *mip*, the protein is suggested to be involved in optimal intracellular infection and full expression of animal virulence. However, as the *mip*⁻ mutant remains competent for intracellular replication after an initial delay in establishment of infection (Cianciotto et al., 1989), Mip seems to play a role in one or more early events in the infective process that determine productive intracellular infection, including inhibition of acidification of phagosome, phagosome-lysosome fusion, recruitment of host intracellular organelles, or provision of nutrients.

Interestingly, the amino acid sequence of the C-terminal region of the Mip protein has a high degree of homology to that of FK-506 binding protein (FKBP) found in various prokaryotic and eukaryotic organisms (Bangsberg et al., 1991). FKBP's possess peptidyl-prolyl *cis/trans* isomerase (PPIase) activity, which is able to catalyse the slow conformational interconversion of prolyl bonds in oligopeptides and proteins *in vitro* (Fischer et al., 1989; Fischer and Schmid, 1990) and *in vivo* (Lodish and Kong, 1991; Colley et al., 1991), and is specifically inhibited by the immunosuppressant FK506 and rapamycin (Liu et al., 1991a). FKBP-drug complexes prevent the transcription of genes involved in T-cell activation and are able to inhibit the clonal expansion of T-cells (Schreiber and Crabtree, 1992; Fruman, et al., 1994). Thus, FK506 and rapamycin are used as potent immunosuppressants in the prevention of allograft rejection. It is apparent that FKBP-drug complexes block translocation of a calcium-dependent NF-AT (nuclear factor of activated T cells) to the nucleus (Flanagan et al., 1991), thereby preventing the transcription of IL-2 mRNA and a number of other mRNAs including c-myc, hsp70, IFN- γ and TNF- α . Also, Liu et al. (1991b) recently demonstrated that calcineurin, a calcium/calmodulin-dependent serine/threonine phosphatase, is a target molecule of FKBP-drug complexes. The results suggest that inhibition of the phosphatase prevents the activation of the cytoplasmic NF-AT.

It was shown that the Mip protein of *L. pneumophila* exhibits PPlase activity and that FK506 and rapamycin inhibit its activity as do also for FKBP of eukaryotic cell (Fischer et al., 1992; Lundemose et al., 1993b). A secondary structure prediction, based on amino acid sequence data, indicated that the C-terminal region of Mip shares main structural elements of five β -strands interrupted by a small α -helix with human FKBP (Michnik et al., 1991). Eight out of ten amino acids participating in the interaction of the human FKBP and FK506 were conserved in the Mip protein. Although the N-terminal domain of Mip does not share homology with any other known protein, this part is predicted to have α -helix configuration. Hence the function of this domain is proposed as such; the extended α -helix of the Mip protein projects from the membrane and forms a rod-like space arm to bridge the enzymatically active C-terminal half carrying the PPlase activity in proximity to a putative partner molecule in the host vacuolar membrane structure.

Mip-like proteins are also produced by strains of *Chlamydia trachomatis*, obligate intracellular bacteria responsible for the blinding ocular disease, trachoma, and for sexually transmitted diseases. The 27-kDa Mip-like protein of *C. trachomatis*, known as L2, was shown to be located in the membrane of reticulate bodies (RB) as well as elementary bodies (EB) (Lundemose et al., 1991). Recent experimental data showed that *C. trachomatis* L2 is a lipoprotein since palmitic acid incorporated into the recombinant chlamydial L2 and globomycin, an inhibitor to signal peptidase II, inhibited processing of the recombinant L2 (Lundemose et al., 1993a). The chlamydial L2 had 35% amino acid identity to that of *L. pneumophila* (Lundemose et al., 1992) and the 110 amino-acids of C-terminal domain showed 41% identity to human FKBP (Maki et al., 1990). Chlamydial Mip-like protein also showed PPlase activity, which was inhibited by either rapamycin or FK506 as seen in the *Legionella* Mip protein (Lundemose et al., 1993b). When chlamydial organisms

were pretreated with FK506 or antibodies against Mip to assess the role of the Mip-like protein in chlamydial infection, infectivity was reduced by 30% for McCoy cells (Lundemose et al., 1993b) and by 91% for HeLa cells (Lundemose et al., 1992). The results suggested that inhibition of the PPlase of Mip-like protein interferes with the early steps of intracellular infection processes.

The *mip* gene from *Coxiella burnetii*, the obligate intracellular rickettsia that causes Q fever, was recently cloned and its nucleotide sequence determined (Mo et al., 1995). The predicted amino acid sequence showed 46% and 30% similarity with Mip proteins of *Legionella* and *Chlamydia*, respectively. Likewise, in *Legionella* and *Chlamydia*, the C-terminal amino acid sequence had over 35% identity to prokaryotic and eukaryotic FKBP and exhibited PPlase activity when overproduced in *E. coli*. Further detection of Mip-related proteins in *Coxiella burnetii* and *Rochalimaea quintana* as well as within *Rickettsia* and *Ehrlichia* species (Cianciotto et al., 1995) gives a strong indication that Mip-like proteins play an important role in host-parasite interactions.

The finding that a Mip-like protein of the protozoan parasite *Trypanosoma cruzi* (TcMip) is involved in cell invasion (Moro et al., 1995) leads to the assumption that FKBP homologues of pathogenic bacteria may represent a new class of pathogenicity factors. The amino acid similarity of TcMip was 25.5% with Mip from *Chlamydia* and human FKBP12 and 29.6% with MIP from *Legionella*. The detection of TcMip in the culture medium of trypomastigotes, the non-dividing form of the parasite responsible for infectivity, clearly indicated that TcMip was secreted by the infective stage of the parasite, and suggested that a putative target molecule for the PPlase activity was on the host cell membrane. The observation that addition of TcMip to cultured mammalian cells enhanced parasitic invasion while antibodies against TcMip greatly reduced infectivity indicated that TcMip, like Mip from other parasites, is involved in the initial infection of host cells.

C. Objectives

Although speculation exists that certain component(s) encoded by X-bacterial plasmids, 96-kDa protein and LPS on symbiosomal membrane produced by X-bacteria may function as fusion-blocking factors, many questions still remain to be answered. The mechanisms of how X-bacteria escape from phagolysosomal destruction and the identity of factors playing essential roles in intracellular survival of X-bacteria within host amoebae are two such issues. Hence, this study was motivated to identify and characterize factors contributing to the survival and multiplication of X-bacteria in amoebae.

The Mip protein was first identified as a virulence factor in *Legionella* and was known to be involved in the initial infection. A Mip-like protein has also been detected in the majority of pathogenic strains including *Chlamydia* and *Coxiella* species. Mip-like proteins thus appeared to be an important factor determining bacterial pathogenicity and host-symbiont interaction. Recent studies analyzing LPS components of the X-bacterial cell wall (Kim & Jeon, unpublished data) and the sequence of the *groELx* heat-shock gene (Ahn et al., 1991) pointed out that X-bacteria may be closely related to *Legionella* species. As a consequence, it seemed reasonable to speculate that X-bacteria contained a Mip-like protein.

A Mip-like protein produced by X-bacteria was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel (2-D) electrophoresis and immunoblot using *Legionella* anti-Mip antibody as a probe. The *mipx* gene was cloned from an X-bacterial genomic library using *mip* gene sequences of *Legionella* species as primers. Its nucleotide and amino-acid sequences were then determined and characterized. A recombinant Mipx protein was overproduced in *E. coli*, purified, and PPLase activity of the C-terminal domain was tested in a coupled assay with α -chymotrypsin as a protease and N-succinyl-

Ala-Ala-Pro-Phe-4-nitroaniide as a substrate peptide. And the possible function of Mipx protein in X-bacteria-amoeba interaction was investigated with experimental infection.

While studying the symbiosomal membrane components as fusion-blocking factors and making monoclonal antibody (mAb) against 210-220 kDa polypeptides on the symbiosomal membrane, it has been found that a set of mAb turned out to recognize the myosin of amoeba throughout cytoplasm as well as on symbiosomal membrane. Therefore, another objective of this study was to characterize the myosin as a host-derived symbiosomal membrane component and its cDNA gene in *Amoeba proteus*. The cDNA nucleotide sequence was completed and deduced amino-acid sequence was analyzed by comparing with those of myosin II genes from various organisms regarding the globular head region and the α -helical coiled-coil tail region. The localization of myosin on symbiosomal membrane was investigated by immunofluorescence and immunoelectron microscopy using an anti-myosin monoclonal antibody as a probe. The isoforms of myosin type II within amoebae were investigated by Northern blot analysis using conserved sequences of the head region containing ATP-binding site or actin-binding site as probes.

CHAPTER II

MATERIAL AND METHODS

A. Materials

For DNA work, restriction enzymes and other DNA-modifying enzymes were purchased from United States Biochemical Co. (USB, Cleveland, OH), Stratagene (La Jolla, CA) or Promega (Madison, WI). Exonuclease III and mung bean nuclease were purchased from Promega. T4 ligase and modified T7 DNA polymerase (Sequenase version 2.0) for DNA sequencing were purchased from USB. *Taq* and *Pfu* polymerase for PCR reactions were obtained from Promega. Total RNA isolation Kit and random primed-labeling kit were purchased from 5'→3' Inc. (Boulder, CO) and Gibco BRL (Grand Island, NY), respectively. For gel electrophoresis, acrylamide, N,N'-methylene-bis-acrylamide, SDS, Tris and Urea were obtained from USB. Ammonium persulfate and TEMED came from BioRad (Richmond, VA). Ampholine (pH 3.5-10 and 5-8) was acquired from LKB (Rockville, MD). For the production of monoclonal antibodies, fetal bovine calf serum and Dubellco's Modified Eagles medium were obtained from Hyclone (Logan, UT). Hypoxanthine-Aminopterin-Thymidine, Hypoxanthine-Thymidine media, and antibiotics were purchased from Gibco-BRL. Polyethylene glycol 4000 came from Sigma Chemical Co. (St. Louis, MO). For indirect immunofluorescence microscopy, secondary antibodies against mouse IgG conjugated with horse-radish peroxidase (HRP) or FITC were purchased from Jackson ImmunoResearch (West Grove, PA). Paraformaldehyde was acquired from Aldrich Chemicals (Milwaukee, WI). For electron microscopy, glutaraldehyde (50% solution) and sodium cacodylate were purchased from EM Science (Fort Washington, PA). Goat anti-mouse IgG antibody

conjugated with 15 nm gold particles were obtained from E.Y. Laboratories (San Mateo, CA). Lowicryl K4M was from Polysciences (Warrington, PA). For autoradiography, X-Omat AR film, developer and fixer for the X-ray film were purchased Kodak Co. (Rochester, NY). Agarose for DNA gel electrophoresis was purchased from FMC (Rockland, MD) and nitrocellulose paper from Schleicher & Schuell Co. (Keene, NH). Immobilized protein A and Ni⁺-resin columns for antibody and protein purification were purchased from Pierce (Rockford, IL) and Novagen Inc. (Madison, WI), respectively. Buffer components and other inorganic chemicals came from Sigma and USB.

B. Organisms

1. *Amoeba proteus*

Two strains of *Amoeba proteus*, D and xD, were used in this study. xD amoebae originated from D amoebae by spontaneous infection with unknown rod-shaped bacteria (Jeon and Lorch, 1967). For mass culture of amoebae, cells were grown in a modified Chalkley's solution (Jeon and Jeon, 1975) with axenically cultured *Tetrahymena pyriformis* as a food organism. Amoebae were grown in Pyrex baking dishes (34 x 22 x 4 cm) and fed *Tetrahymena* for 2 hr every other day. Cultures were washed with fresh culture medium after feeding period to prevent overfeeding. *Tetrahymena* were grown on 2% proteose peptone, 0.2% liver concentrate, and vitamin concentrate (Goldstein and Ko, 1976).

For stock culture of amoebae, *Colpidium* sp. were used as food organisms (Lorch and Danielli, 1953). To glass pie dishes (15.2 cm in diameter), ten wheat grains (boiled and dried) and approximately 300 healthy amoebae were added with *Colpidia* filtered through an absorbent cotton layer in Chalkely's solution. The cultures were renewed once a month.

2. HeLa and Vero (monkey kidney) cells

Cells were grown in complete DME containing 10% calf serum with 5.5% CO₂ supply in 75 cm² tissue culture flasks. For renewal when cell growth reached confluency, the culture supernatant was discarded and cells were treated with 10 ml of trypsin-EDTA (1X, Flow Laboratories) for 3 min at 37° C. The flask was then shaken gently to detach cells from culture flask. Cells were collected, centrifuged for 1 min at 170 g, and then suspended in 10 ml of incomplete DME. Following an additional centrifugation to remove trypsin-EDTA, cells were resuspended in 1 ml complete DME as above, and 0.5 ml of the suspension was inoculated into a new flask containing 10 ml of complete DME with 10% calf serum.

3. Bacteria

Various strains of *Escherichia coli* including XL1-Blue, BB4 and BL21 were used for this study. pBluescript, pET-30a, pET-30b, and pTA were used as cloning or expression vectors. In general, bacteria were cultured by inoculating single isolated colonies from stock plates to 3 ml of Luria-Bertina (LB) medium with or without selective antibiotics and incubated overnight with vigorous shaking at 225 rpm in a rotary shaker. Mini-scale preparation for DNA analysis was done with 3 ml of overnight culture. Bacterial cells were routinely harvested by centrifugation for 2 min at 12,000 rpm in a Eppendorf centrifuge. When a large-scale culture was needed, 3-ml overnight culture was further inoculated into 500 ml of culture media. After washing once with TE buffer (10-mM Tris-HCl, PH 8.0, 1-mM EDTA), bacteria were processed to the next step or kept frozen at -20° C for further experimental use.

4. X-Bacteria

Symbiotic bacteria were isolated from 3-day starved xD amoebae. Harvested amoebae were suspended in 5 volumes of 20-mM Tris-HCl buffer (pH 7.4) containing

2% Triton X-100, frozen and thawed. Vortexed cell suspensions were frozen once more and thawed to break down any intact symbiosomes. The supernatant fraction was saved following centrifugation for 5 min at 170 g. The pellet fraction was suspended in the same buffer and subjected to the same freezing and thawing process again to collect the supernatant fraction. The pooled supernatants were layered over a 30/50% Percoll step gradient and centrifuged for 10 min at 10,000 g. X-Bacteria were collected from the bottom of the gradient and washed twice with the same buffer to remove Percoll. Purified X-bacteria were stored at -70° C until used.

C. Nucleic Acid Isolation

1. Isolation of X-bacterial genomic DNA

Symbiotic bacteria were suspended in X-bacteria lysis buffer (50-mM Tris-HCl pH 8.0, 150-mM glucose, 10-mM EDTA) and incubated for 10 min at 37° C. Then Proteinase-K was added to the bacterial suspension to a final concentration of 500 µg/ml. Samples were incubated for another 30 min at 37° C, followed by addition of SDS to a final concentration of 2%. After further incubation for 1 h at 60° C, samples were phenol, chloroform-extracted and ethanol-precipitated in the presence of 3-M sodium acetate (pH 7.0). The final pellet was washed once with 70% ethanol, dried, and dissolved in TE buffer. RNAs were removed by incubating DNA sample solution with RNase A (10 µg/ml, final conc.) for 15 min at 37° C.

2. Isolation of plasmid DNA

Plasmid DNA was isolated by the alkaline lysis method (Birmboim and Doly, 1979). Cells were thoroughly resuspended in Sol I (50-mM glucose, 10-mM EDTA, 25-mM Tris-HCl, pH 8.0). Two volumes of Sol II (0.2-N NaOH and 1% SDS) were

added and the mixture gently inverted 2 - 3 times followed by incubation on ice for 5 min. One and one-half volumes of Sol III (5-M potassium acetate, pH 4.8) were added with gentle mixing and incubation continued for another 5 min. After centrifugation, the supernatant was collected and DNA extracted with phenol and chloroform, followed by ethanol precipitation and addition of 0.1 volume of Sol IV (3-M sodium acetate). The plasmid pellet obtained was resuspended in TE or water and treated with RNase A at a concentration of 10 µg/ml for 15 min at 37° C. Large amounts of plasmid DNA for mung bean deletion were prepared by CsCl ultra-centrifugation.

3. Isolation of total cellular RNA from amoebae

Total RNA was prepared from 0.5-ml packed amoebae by the total RNA isolation kit (5'→3' Inc., Boulder, CO) according to manufacturer's methods. Isolated RNA pellets were dissolved in water pretreated with 0.1% DEPC. The amount of RNAs was measured spectrophotometrically at 260 nm.

D. PCR Amplification

The *mip* gene (*mipx*) of X-bacteria was identified by Polymerase Chain Reaction (PCR) using an X-bacterial genomic library (Park and Jeon, 1990) as a template and vector, and specific sequences of *Legionella mip* gene as primers. PCR was performed for 35 cycles under the following conditions; denaturation for 1 min at 94° C, annealing for 1 min at 60° C and extension for 2 min at 72° C. The PCR product was cloned into the pTA vector and subjected to 'nested deletion' by *ExoIII* and mung bean nuclease for sequence analysis as described below. The 5' most ~500 nucleotides of amoeba myosin heavy chain (MHC) gene were PCR-

amplified using amoeba cDNA library (Choi et al., 1992) under the same conditions and the PCR product was ligated into the *Sma*I site of pBSK⁺ vector for further study.

E. Sequence Analysis

1. Construction of deletion series

An *ExoIII*/mung bean nuclease combination was used to generate the nested deletion series (Henikoff, 1984). *SacI*/*Bam*HI double-digested DNAs (50 µg) were phenol extracted, precipitated, dried and dissolved in the *ExoIII* nuclease reaction buffer. After adding *ExoIII* nuclease to the reaction tube, 25 µl of sample was taken out of the reaction tube at each time point (60-sec intervals at 30° C) and transferred to a tube containing 175 µl of mung bean nuclease buffer. Further reaction of *ExoIII* nuclease was stopped by placing tubes on dry ice and then the enzyme was heat-killed for 15 min at 68° C. Mung bean nuclease was added to tubes and incubated for 30 min at 37° C. At the end of incubation, 4 µl of 20% SDS, 10 µl of Tris (pH 9.5), 20 µl of 8-M LiCl, and 250 µl of phenol:chloroform (1:1) were added. After centrifugation, the aqueous phase was ethanol precipitated. The DNA pellet was washed with 70% ethanol, dried and dissolved in 10 µl of sterile distilled water. One µl was used for a standard ligation and 3 µl were used for rapid analysis of the amount of deletion.

2. Transformation

Transformation was done following TSS method (Chung et al., 1989) with some modifications. A 0.2-ml sample of the overnight bacterial culture was inoculated into 3 ml of LB medium and grown until the OD₆₀₀ reached 0.6. 1-ml cells was harvested and resuspended in 0.1 ml of TSS (1% bactotrypton, 0.5% yeast-

extract, 0.25-M NaCl, 1% PEG, 0.25% DMSO and 50-mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.5). 1- μl of ligated DNA was added and tube was kept on ice for 40 min. 0.9 ml of 20-mM glucose-TSS was added to the tube and incubated with gentle agitation for 1 hr at 37° C. Cells were pelleted by centrifugation for 30 sec, resuspended in 0.15 ml of LB medium and were plated on agar plates containing appropriate antibiotics.

3. Sequencing reactions

The DNA sequence was determined by the dideoxynucleotide chain termination method (Sanger et al., 1977) using Sequenase (USB). For a single reaction, 2 - 4 μg of double-stranded DNA were used. The sequencing gel was prepared by dissolving 42.05 g of urea (8-M) in the mixture of 20 ml of 5X TBE (45-mM Tris-borate, 1-mM EDTA), 12.5 ml of 40% polyacrylamide and 37.5 ml of distilled water. The gel solution was poured on a large glass plate (41.5 X 35 X 0.3 cm) and a siliconized small glass plate (39 X 35 X 0.3 cm) was placed on it for gel polymerization (Lang and Burger, 1990). The sequencing gel was electrophoresed for 2.5 hr at 35 W. At the end of running, the small plate was taken off and the gel was treated with 5% methanol/5% acetic acid for 15 min for fixation and for removal of urea. All DNA sequences from manual sequencing were reconfirmed by automatic sequencing (Applied Biosystems, Model 373A) using synthetic oligonucleotides as primers.

F. Gel Electrophoresis and Blot Transfer of Nucleic Acids

1. Southern blotting

X-Bacterial genomic DNA digested with various restriction enzymes were electrophoresed at 5 V/cm in 0.7% agarose gels. Gel and chamber buffer was TAE

(40-mM Tris-acetate, 1-mM EDTA). After electrophoresis, gels were treated with denaturation solution (0.5-N NaOH, 1.5-M NaCl) for 30 min followed by two changes of neutralizing solution (1.5-M NaCl, 1-M Tris pH 7.4) for 45 min. The DNA was blotted onto a positively charged nitrocellulose membrane using 10X SSC (1X SSC: 150-mM NaCl, 15-mM sodium acetate) as a transfer buffer. The DNA was fixed to the membrane and sandwiched between two sheets of 3 MM paper by baking for 2 hr at 80° C in a vacuum oven.

2. Southern hybridization

The blots were prehybridized at 42° C in 50% formamide, 6X SSC, 1% SDS and 100 µg/ml denatured salmon sperm DNA for 2 hr. Hybridization was then carried out under the same conditions with radioactive probes for 24 hr (Sambrook, et al., 1989). After hybridization, blots were washed in two changes of 2X SSC for 20 min at room temperature and then 2X SSC + 1% SDS for 30 min at 65° C followed by 0.1X SSC for 30 min at room temperature. The filters were covered with Saran wrap and exposed to X-ray film at -70° C for appropriate lengths of time.

3. Northern blotting

RNA samples were denatured in a sample mixture containing 50% formamide at 68° C and electrophoresed at 5 V/cm in a 1% agarose gel containing 6.6% formaldehyde. Gel and chamber buffer was MOPS [40-mM 3-(N-Morpholino) propanesulfonic acid, 10-mM sodium acetate, 1-mM EDTA]. After electrophoresis, gels were soaked in DEPC-water for 30 min to remove formaldehyde. The RNA was blotted onto positively charged nitrocellulose membranes and fixed as described above.

4. Northern hybridization

The filters were prehybridized at 42° C in 5X Denhardt's [1X Denhardt's: 0.02% ficoll, 0.02% polyvinyl pyrolidone, and 0.02% bovine serum albumin fraction V (Sigma)], 50% formamide, 0.8-M NaCl, 0.1-M PIPES, pH 7.0, and 100 µg/ml

denatured salmon sperm DNA for 4 hr. Hybridization was carried out under the same conditions for 24 hr (Sambrook et al., 1989). After hybridization, blots were washed in 4 changes of 2X SSC, 0.05% sodium pyrophosphate, 0.05% SDS for 40 min at room temperature, followed by two changes of 0.2X SSC, 0.05% sodium pyrophosphate, 0.05% SDS for 30 min at 60° C. The Blots were covered with Saran wrap and exposed to X-ray film at -70° C for appropriate lengths of time.

G. Gel Electrophoresis and Related Techniques of Protein

1. Polyacrylamide-gel electrophoresis (PAGE)

One-dimensional electrophoresis was carried out according to Laemmli (1970). The separating gel solution containing 10% acrylamide, 0.375-M Tris-HCl (pH 8.8) and 0.1% SDS was polymerized by the addition of 0.025% TEMED and 1% ammonium persulfate. The stacking gel was 4% acrylamide gel in 0.125-M Tris-HCl (pH 6.8) and 0.1% SDS. A 20-µg protein sample was mixed with SDS-sample buffer containing 0.125% Tris-HCl (pH 6.8), 2% SDS, 5% sucrose, 5% β-mercaptoethanol and 0.0025% bromophenol blue and then boiled for 2 min just prior to application to each well.

Two-dimensional electrophoresis was carried out according to O'Farrell (1975) with slight modifications. Proteins were mixed with an equal volume of lysis buffer (9.5-M urea, 2% Ampholine, and 5% β-mercaptoethanol) and applied to the top of a tube gel (3 mm in inner diameter, 14 cm in length) containing 4% acrylamide, 9.5-M

urea, 5% β -mercaptoethanol, and 2% Ampholine (pH 3.5-10). Isoelectrofocusing (IEF) was performed for 30 min at 300 V, 30 min at 400 V, 12 hr at 600 V, followed by 2 hr at 800 V. The upper and lower tank buffers were 50-mM NaOH and 25-mM phosphoric acid, respectively. After electrophoresis of the first dimension, gels were removed from tubes and incubated in treatment buffer (0.062- M Tris-HCl, pH 6.8, 2% SDS 10% glycerol, and 5% β -mercaptoethanol) with slow shaking for 30 min, placed on top of a slab gel, and sealed with 1% agarose in the treatment buffer. The 8-14% gradient gels were run for 6 hr at 150 V and either stained with Coomassie blue (CB) or immuno-blotted.

2. Coomassie blue (CB) staining

Gels were stained for 30 min in a solution containing 50% methanol, 10% acetic acid, and 0.1% CB (Fairbanks et al., 1971). The gels were destained with 50% methanol and 10% acetic acid for 1 hr, followed by 10% methanol and 5% acetic acid with several changes of the solution.

3. Electroelution

Protein samples (~20 mg) were applied to a 3-mm thick 10% slab gel without a comb and electrophoresis was performed for 7 hr at 50 mA per gel. After electrophoresis, gels were stained for 1 hr in a solution containing 0.1% CB, 50% methanol, and 10% acetic acid and destained for 1 hr in 50% methanol and 10% acetic acid. Desired polypeptide bands were excised with a razor blade and neutralized for 30 min in 0.1-M Tris-HCl (pH 6.8). The sliced gel pieces were applied to an electroelution column whose tip was sealed with 1% agarose and the elution of proteins was performed for 20 hr at 150 V. Eluted proteins were dialyzed against distilled water overnight at 4° C. Six volumes of cold acetone were added to the dialyzed protein solution and the solution was kept for 1 hr at -20° C prior to centrifugation (Hager and Burgess, 1980).

4. Western blotting

Proteins separated by SDS or 2-D electrophoresis were transferred onto nitrocellulose membranes by Towbin's transfer method (1979) using 3 sheets of wet 3 MM paper, gel, and nitrocellulose membrane. And then 3 sheets of wet 3 MM membrane were placed inside a gel cassette in the order. For the 10% SDS gel, the protein transfer took 2 hr at 200 mA. The transfer buffer was composed of 0.5-M Tris and 0.7-M glycine. After protein transfer, blots were blocked with 2.5% non-fat dry milk in PBS for 1 hr and rinsed with three changes of PBS at 10-min intervals. The blots were then incubated with the culture supernatant of hybridoma cells or ascites fluids (diluted in PBS, 1:500) for 1 hr and washed as before. The blots were treated with goat anti-mouse IgG conjugated with horse-radish peroxidase (HRP) for 1 hr. The washed blots were developed in a substrate solution containing either 30 mg of 4-chloro-naphtol, 30 μ l of H₂O₂ (30%), 10 ml of methanol in 50 ml PBS or 30 mg of diaminobenzidine (DAB), 30 μ l of H₂O₂ in 50 ml PBS (De Blas and Cherwinski, 1983; Tsang et al., 1989).

H. Peptidyl Prolyl *Cis/Trans* Isomerase Assay

The assay was performed by routine procedures (Standaert et al., 1990; Tropschug et al., 1990). Enzyme activity was measured in a coupled assay with chymotrypsin on the basis of the ability of the protease to cleave the peptide substrate only when the Ala-Pro bond is in the *trans* conformation (Schreiber, 1991). Briefly, a stock protein solution of recombinant Mipx (0.5 mg/ml) was diluted 10-fold with the assay buffer (100-mM Tris-HCl, pH 7.4) and, immediately before assay, further diluted to 940 μ l in a 1.5-ml microcentrifuge tube. 30 μ l of peptide substrate [(N-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide, 2.0-mM in DMSO, (Sigma))] was added

permeabilized in cold methanol (-20°C) for 5 min, and then air dried. Amoebae were incubated with culture supernatants of hybridoma cells or ascites fluids for 30 min at room temperature. The cells were then washed in three changes of PBS at 5-min intervals and further processed with the goat anti-mouse IgG secondary antibody conjugated with FITC for 30 min. The labeled amoebae were washed 3 times with PBS and mounted with a mounting solution containing 90% glycerol, 1 mg/ml *p*-phenylenediamine, and 10% PBS (Platt and Michael, 1983), and examined under a Leitz epifluorescence microscope. When only a few samples were handled, amoebae were collected in Syracuse watch glasses and media was removed by aspiration. The cells were fixed and permeabilized with cold methanol for 5 min. For antibody staining, amoebae were hydrated in PBS for 5 min and processed further as described above.

2. Tissue culture cells

Cells were inoculated in 35-mm tissue culture dishes containing preautoclaved glass coverslips (22 X 22 mm) and grown to 80% confluency. The coverslips were taken out and washed with PBS in Columbia jars. Cells were then fixed with 3% paraformaldehyde in PBS for 10 min, washed with PBS, and permeabilized with cold methanol for 5 min. For antibody staining, the process was followed as described above.

K. Immuno-Electron Microscopy

1. Immunoperoxidase method

Amoebae were collected by centrifugation for 1 min at 170 g, fixed and permeabilized with cold methanol (-20°C) for 5 min. After washing 3 times with PBS,

amoebae were treated with mAbs for 1 hr at room temperature, washed again, and further incubated with HRP-conjugated goat anti-mouse IgG for 1 hr. After washing 3 times with PBS, amoebae were postfixed with 1% glutaldehyde in 0.1-M sodium phosphate buffer (pH 7.2) for 30 min at 4° C to minimize 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 DAB and 0.0001% hydrogen peroxide for color developing. The reaction was allowed to proceed until cells became dark brown, as monitored with a light microscope. Amoebae were postfixed with 1% osmium tetroxide in 0.1-M sodium cacodylate buffer (pH 7.2), dehydrated with ethanol, and embedded in Epon (Spurr, 1969). Gold-colored thin sections were collected on copper grids and stained with uranyl acetate for 30 min and with lead citrate for 3 min, and then observed under a Hitachi H-600 electron microscope.

2. Immunogold method

Packed xD amoebae were fixed in a solution containing 3% paraformaldehyde, 0.3% glutaldehyde, and 10% sucrose in 0.1-M sodium phosphate (pH 7.2) for 1 hr. After washing twice with the same buffer, amoebae were dehydrated in a series of dimethylformamide (DMF) solutions; 10 min in 50%, 15 min in 70%, 15% in 90%. Then, amoebae were infiltrated and embedded in Lowicryl K4M as follows (Altman et al., 1984): 15 min in 2:1 (DMF:Lowicryl), 30 min in 1:1, 30 min twice in Lowicryl only. Polymerization was done at 4° C with exposure to UV light (360 nm) overnight. Gold-colored thin sections were collected on nickel grids and treated with 1% BSA for 1 hr to block non-specific binding. Sections were treated with the primary antibody for 1 hr and washed extensively with PBS followed by treatment with goat anti-mouse IgG antibody conjugated with colloid gold (15 nm). The sections were stained with lead citrate for 3 min and observed in a Hitachi H-600 electron microscope.

L. Production of Monoclonal Antibodies

1. Media preparation

A. Incomplete Dulbecco's modified Eagle's medium

One package of powdered medium (Gibco-BRL) was dissolved in 900 ml of distilled water and 3.7 g of sodium bicarbonate was added to the solution. The medium was adjusted to pH 6.95 with 1-N HCl, added up to 1 L with distilled water, and sterilized by passing through a 0.45- μ m filter unit (Corning).

B. Complete Dulbecco's modified Eagle's medium

To make 500 ml of complete DME, 100 ml of fetal bovine serum and 5 ml of penicillin-streptomycin solution (10,000 unit, Gibco-BRL) were added to 400 ml of incomplete DME.

C. Hypoxanthine-Thymidine (HT) and Hypoxanthine-Aminopterin-Thymidine (HAT) medium

To make 500 ml HT and HAT medium solution, 100X concentrated HT and HAT stock solution (Gibco-BRL) were mixed with 500 ml of complete DME to make HT and HAT medium, respectively.

D. Cell freezing medium

To make 100 ml of cell freezing medium, 10 ml of dimethyl sulfoxide (DMSO) was added to 90 ml of fetal bovine serum.

2. Antigen preparation and injection

Protein samples were injected into each female BALB/c mouse (6-8 weeks

old) intraperitoneally with complete Freund's adjuvant. The first injection was followed by three booster injections at 2-week intervals. The final injection was given three days prior to cell fusion.

3. Preparation of cells for fusion

A. Feeder layer cells

One day before the cell fusion, a mouse was killed by cervical dislocation and its abdominal skin was carefully removed. 5 ml of sucrose solution (11.6%) was injected into the peritoneal cavity and about 3 ml of injected solution was recovered by a syringe with an 18-gauge needle. The collected peritoneal cells were pelleted by centrifugation for 3 min at 750 g and suspended in 60 ml of HAT medium. 0.5-ml suspension was dropped into each well of five 24-well plates. The plates were kept in a 5.5% CO₂ incubator at 37° C.

B. Myeloma cells

Five days before cell fusion, frozen SP2/0-Ag-14 cells were taken out of a liquid nitrogen tank, thawed in a 37° C water bath and transferred to a 15-ml tube. 10 ml of complete DME was then added over a period of 5 min with gentle stirring. The cells were spun down for 3 min at 650 g, suspended in 10 ml of complete DME, and grown at 37° C. The medium was renewed every other day. About 5×10^7 cells were used for each cell fusion.

C. Spleen cells

A mouse injected with antigens was killed and the spleen was removed under sterile conditions. The spleen cells were released into 10 ml incomplete DME by squeezing the spleen with fine forceps and transferred into a 15-ml tube. The tube

was allowed to stand for 2 min at room temperature and 7 ml of the cell suspension from the top were collected. After centrifugation for 3 min at 650 g, the pellet was re-suspended in 50-ml of incomplete DME. The cells were washed once more with incomplete DME, and the cell number was adjusted to 3×10^7 for each fusion.

4. Cell fusion

Prepared SP2 and spleen cell suspensions were mixed and centrifuged for 3 min 170 g. The pellet was washed with 30 ml of incomplete DME by centrifugation for 3 min at 170 g. The supernatant was removed thoroughly because the final concentration of PEG is critical for cell fusion. Pelleted cells were mixed by agitation and 1 ml of PEG (40% in incomplete DME) was added slowly over 1 min with constant swirling at 37° C. The fusion reaction was continued for 2 min at 37° C in a water bath. To stop the fusion reaction, 1 ml of incomplete DME was added carefully over 1 min and 2 ml over 1 min at 1-min intervals with constant swirling. For a period of 10 min, a total 30 ml of incomplete DME was added and the tube was incubated for 5 min at 37° C. PEG was removed by centrifugation for 3 min at 170 g and the cells were suspended in 20 ml of HAT medium by swirling. After centrifugation as before, cells were resuspended in 120 ml of HAT medium, and 1 ml of the cell suspension was placed into each well of five 24-well plates which contained feeder layer cells. Four days after fusion, 1 ml of HAT medium was aspirated and 1 ml of HT medium was added to each well. Three days later, most of the supernatant was removed and cells were fed with new HT medium. Hybridoma colonies began to be detected 4 days after fusion on an inverted microscope, and cells were typically ready to be screened for antibodies two weeks after fusion. Positive clones screened by immuno-dot blotting, Western blotting or indirect immunofluorescence microscopy were transferred to 6-well plates and finally grown in tissue culture flasks (75 cm²). The fusion step was most important for the production of monoclonal antibodies.

5. Cloning of hybridoma cells

Frozen hybridoma cells which had been identified as positive cells were thawed and cultured in complete DME at 37° C. The log-phase cells were diluted with PBS and their number was determined by using a Neubauer cell counter, and adjusted to 15 cells per ml by serial dilution with complete DME. One drop of cell suspension was placed into 96-well plates containing feeder cells and two more drops of complete DME were added into each well. The wells containing a single colony were selected on an inverted microscope after 4 days and the cells in selected wells were transferred to 24-well plates after one week. The culture supernatants were screened as described before.

6. Production of ascites fluids

To produce ascites fluids, mice were first injected with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane) in order to induce tumors. One week later, 10^7 cells in 0.5-ml sterilized PBS were injected into mouse intraperitoneally. After approximately one week, an accumulation of ascites fluid was checked by abdominal swelling. The mouse was anesthetized with metofane (2, 2-dichloro-1, 1-difluoro-ethyl methyl ether) and ascites fluid was collected in a centrifuge tube by inserting a 18-gauge needle into the peritoneal cavity. Three or four more tapplings were carried out at 2-3 day intervals. The fluid was centrifuged for 10 min at 7000 g, and stored at -70° C.

N. Production and Purification of Protein

For the expression of *mipx* in *E. coli.*, *mipx* was introduced into the *EcoRI/EcoRV* site of the pET-30a vector and transformed in the BL21 (DE3) strain of

E. coli. Mipx, as a fusion protein containing a His-tag stretch, was overproduced by induction with 1-mM IPTG. The cells were harvested 3 hr after the addition of IPTG to the culture when the OD₆₀₀ reached 0.7 and then sonicated in 20-mM Tris-HCl buffer (pH 7.4). The purification of overproduced protein was achieved by Ni²⁺-affinity column chromatography. The supernatant after centrifugation was filtered through a 0.2- μ m pore syringe-membrane to prevent clogging of the resin. The protein solution was loaded onto a polypropylene column packed with 2.5-ml of Ni²⁺-charged resin at a flow rate of 25 ml/hr. After unbound proteins were washed away, the target protein was recovered by elution buffer (0.4-M imidazole, 0.2-M NaCl, 10-mM Tris-HCl, pH 7.9). The eluted protein was then dialyzed against 100-mM Tris-HCl buffer (pH 7.4) overnight and concentrated appropriately by using Centricon-10 (Amicon). The protein concentration was determined by measuring the optical density at 280 nm and the purity of a preparation was checked by SDS-PAGE.

O. Purification of Antibodies

For purification of mAbs, 50 ml of culture fluid from hybridoma cells were centrifuged for 25 min at 15,000 g and the supernatant was filtered through a 0.2- μ m pore syringe-membrane. Subsequently, the filtered solution was applied to the top of a protein A-coated agarose column (1-ml packed volume, Pierce). The column was then washed extensively with PBS until the absorbance of unbound protein 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. The solution containing antibodies was dialyzed overnight against 20-mM Tris-HCl buffer and concentrated by using Centricon-10 to appropriate concentration. All procedures were performed at 4° C.

CHAPTER III

RESULTS

A. Mip-like Protein in X-Bacteria

1. Detection of Mip-like protein

For checking to see whether X-bacteria contain Mip-like protein, X-bacteria isolated from xD amoebae were sonicated and total proteins were subjected to SDS-PAGE, followed by electrophoretic transfer of polypeptides to a nitrocellulose membrane. A polyclonal antibody against Mip of *Legionella pneumophila* was used as the primary antibody for immunodetection of a Mip-like protein in X-bacteria. Fig. 1 shows that X-bacteria produced a Mip-like protein with a molecular weight of 30,000. When 2-dimensional gel electrophoresis and immunoblotting were performed with X-bacterial protein, a single positive signal was detected in the basic region (Fig. 2B), indicating that the Mip-like protein in X-bacteria might be a basic protein. Since the Mip protein of *Legionella* and *Chlamydia* is known to be a membrane protein, X-bacteria were fractionated with detergents to see whether the Mip-like protein of X-bacteria was also a membrane protein. The pellet, after sonication, was first treated with 2% Triton X-100 to extract cytoplasmic membrane proteins. After centrifugation, the pellet was treated with 1% sarcosyl solution to extract outer membrane proteins. Fig. 3 (Lane 4 & 5) indirectly shows that the Mip-like protein in X-bacteria (Mipx) is localized on the membrane.

2. Nucleotide and deduced amino-acid sequence of *mipx* gene

Following detection of a Mip-like protein (Mipx) in X-bacteria by SDS-PAGE and Western blot, PCR was performed with synthetic degenerate

Figure 1. Coomassie-blue-stained SDS gel (A) and corresponding immunoblot (B) of Mip-like protein in X-bacteria.

Lane 1. Whole-cell proteins of D-amoebae

Lane 2. Whole-cell proteins of X-bacteria

Polyclonal antibodies against *Legionella* Mip were used as a primary antibody.

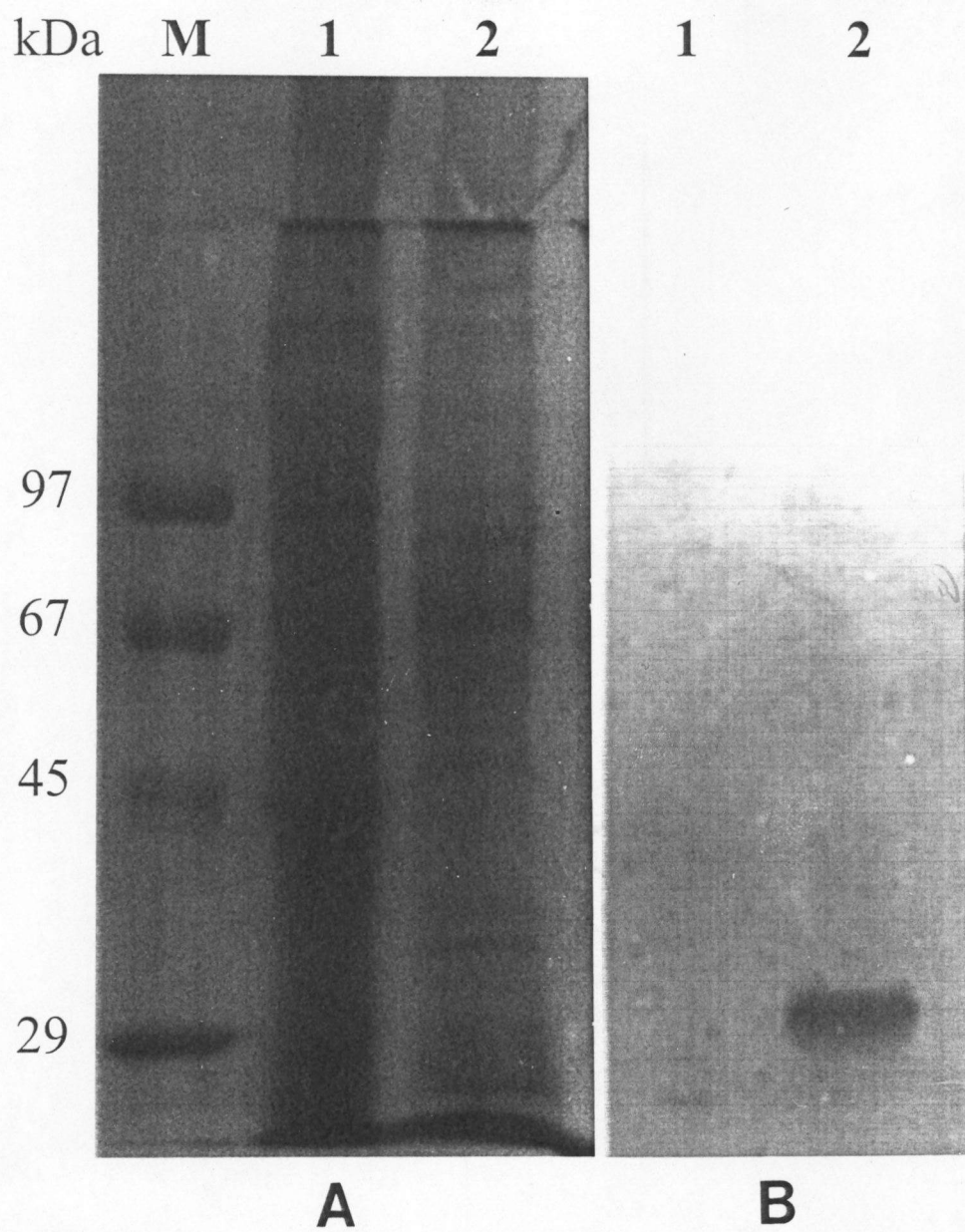
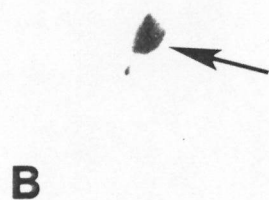
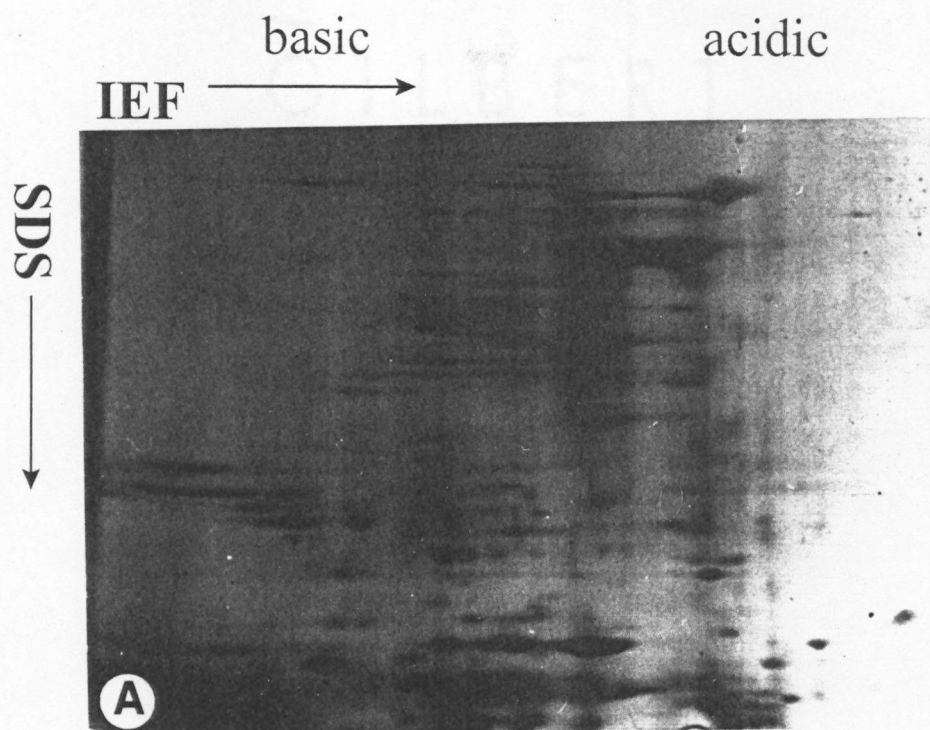


Figure 2. Coomassie-blue-stained two-dimensional gel (A) and corresponding immunoblot (B) of Mip-like protein in X-bacteria.

Arrow (B) indicates a Mip-like protein that positions in the basic region.



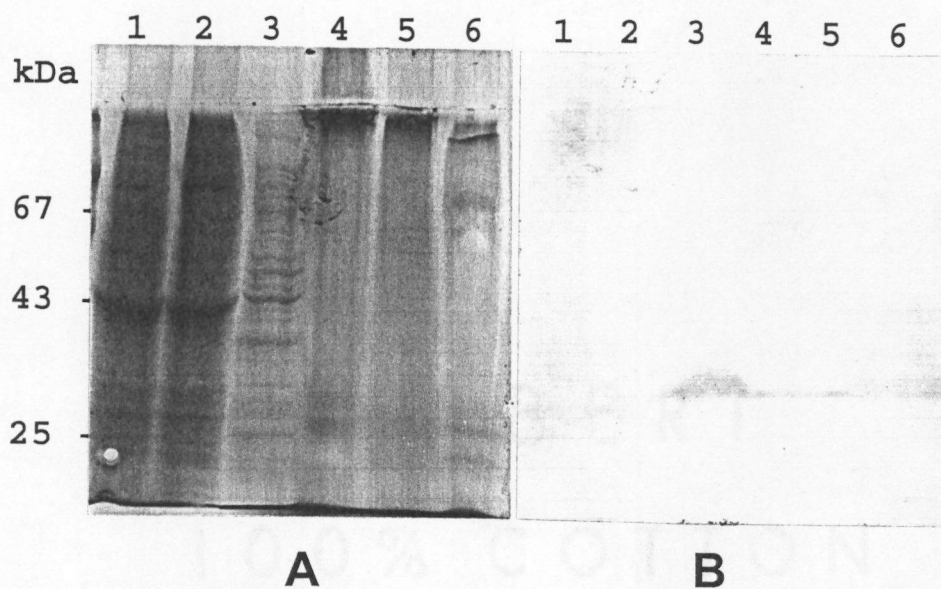


Figure 3. Coomassie-blue-stained SDS-gel (A) and corresponding immunoblot (B) of detergent-fractionated X-bacterial proteins.

- Lane 1. Whole-cell proteins of D-amoebae
 2. Whole-cell proteins of xD-amoebae
 3. Whole-cell proteins of X-bacteria
 4. 2% Triton X-100 extract of sonicated X-bacteria
 5. 1% Sarcosyl extract of sonicated X-bacteria
 6. Sarcosyl-extracted pellet of X-bacteria

oligonucleotides as primers under standard conditions. Synthetic nucleotides were designed from conserved sequences of the *mip* genes of *Legionella micdadei* and *L. pneumophila*. The forward primer was 5'-GGGGATTGTTTATGAAGATGAAA/GA-3', which contained the ribosome-binding sites and start codon, and the reverse primer was 5'-CCAATA/TGGT/GCCGCCAACGCT-3'. Amplified products of the PCR reaction were examined in a 0.8% agarose gel, and a single 700-bp PCR fragment, which was consistent with expected molecular size, was resolved from a λ ZAP II genomic library of X-bacteria (Fig. 4, Lane 1 & 2). Gel-eluted PCR fragments were filled up with dNTP and T4 DNA polymerase prior to cloning into pBSK⁺ vector to analyze its DNA sequence. Amplified DNA was found to be a fragment of *mip*-like gene (*mipx*) by nucleotide sequencing, and the gel-eluted PCR fragments were radiolabeled with ³²P-dCTP by random-primed synthesis and hybridized to X-bacterial chromosomal DNA digested with various restriction endonucleases under high stringency conditions. Each lane revealed a single prominent band (Fig. 5), indicating that X-bacteria chromosomal DNA contained a *mip*-like gene.

PCR was done further by using internal sequences of the *mipx* gene and library vector-specific sequences as primers to find the complete open reading frame (ORF) of *mipx* gene. For the upstream region, a vector-specific T7 primer (forward), 5'-AATACGACTCACTATAG-3' and an internal sequence primer (reverse), 5'-GTCAGGTGCAGCGCTTGC-3', were used. For the 3'-end region of *mipx* gene, a vector-specific T3 primer (reverse), 5'-ATTAACCCCTCACTAAAG-3', and an internal sequence primer (forward), 5'-TGGACTGAGGCACTGCAA-3', were used. Nucleotide sequencing was performed by the dideoxychain-termination method using Sequenase (U. S. Biochemical, Cleveland, OH). The DNA sequence was further confirmed by an automatic sequencer (Applied biosystem: Model 373A).

The complete nucleotide sequence of *mipx* revealed that it contained one ORF of 729 bp encoding 243 amino acids (Fig. 6). The potential ribosome-binding

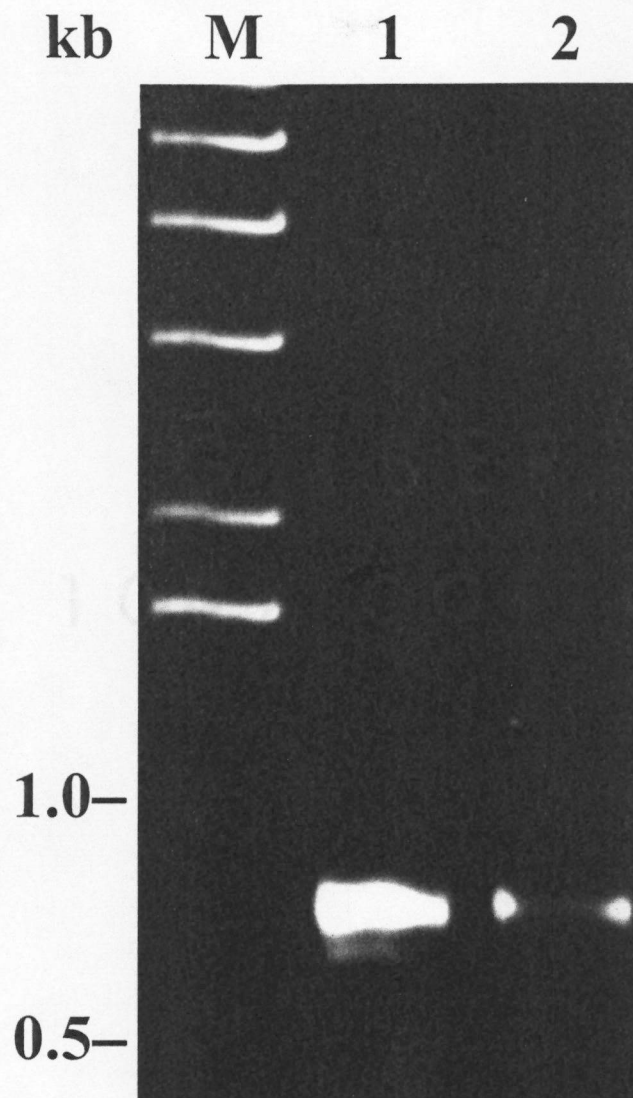


Figure 4. The *mip*-like gene of X-bacteria amplified by PCR.

Lane M. 1-kb DNA ladder

Lanes 1 & 2. PCR products

Figure 5. Southern-blot analysis of X-bacterial chromosomal DNA.

B, *Bam*HI; **E**, *Eco*RI; **H**, *Hind*III; **K**, *Kpn*I; **S**, *Sal*I

X-Bacterial chromosomal DNA digested with various restriction enzymes was hybridized with a radiolabeled PCR fragment under high stringency conditions. The results showed the presence of a *mip*-like gene in X-bacteria.

B E H K S

Kb
6.6 -

4.4 -

2.3 -

2.0 -

Figure 6. Complete nucleotide and deduced amino-acid sequences of *mipx* in X-bacteria.

The ORF of *mipx* gene has 732 nucleotides and encodes Mipx protein of 243 amino acids. The putative ribosome-binding site (**RBS**) and **EcoRV** enzyme site (GATACA) are underlined and highlighted. The start (**ATG**) and stop (**TAA**) codons are in bold types. The primers used for PCR are also underlined with directions.

GCGAATTGGGCCCTCTAGAT -61

RBS

GCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTGGGCATTGTTT -1

forward

ATGAAGATGAGACTGGTCGCTGCAACTGTCATAGGTTTGGCAATGTCAACGACAATGGCT 60

M K M R → L V A A T V I G L A M S T T M A 20

GCAAGCGCTGCACCTGACGCAGCAACTACTGCGTCATCTGCAACATCATTGACTACTGAT 120

A S A A P D A A T T A S S A T S L T T D 40

ATGGATAAGCTGTCTTACAGTATCGGCGCTGATTTAGGTAAGAATCTCAAAAAACAGGGT 180

M D K L S Y S I G A D L G K N L K K Q G 60

ECORV

ATTGATATCAGTCCTGCTGCGATGGCCAAGGGGTACAAGACGGTATAAGCGGTGGCCAA 240

I D I S P A A M A K G L Q D G I S G G Q 80

TTGTTGTTGACTGAGCAGCAAATGAAGGATGTGTTGAATAAATTTCAAAAAGATTTAATG 300

L L L T E Q Q M K D V L N K F Q K D L M 100

GCGAGACGAACGGCTGATTTTAATAAAAAGGCTGAAGAAAATAAAACAAAGGGCGACTCT 360

A R R T A D F N K K A E E N K T K G D S 120

TTTTTAACCAAAATAAAGGAAAAGACGGCGTAGTCAGTTTGCCAAGTGGTTTGCAGTAT 420

F L N Q N K G K D G V V S L P S G L Q Y 140

AAGGTCATTCAAAAAGGCGATGGGGCAAAACCTTCCAAGAAGACACAGTGACTGTCGAC 480

K V I Q K G D G A K P S K E D T V T V D 160

TATACGGGTACATTACTTGATGGCCAAGTTTTTGATACGACTGAAAAACGGGTAAGCCT 540

Y T G T L L D G Q V F D T T E K T G K P 180

GCTACGTTTAAAGTATCACAGGTCATTCCTGGCTGGACTGAGGCACTGCAATTAATGCCC 600

A T F K V S Q V I P G W T E A L Q L M P 200

GCAGGCTCTACCTGGGAAGTGTTTGTTCCTGCAGGTCTTGCTTATGGTCCGCGTAGCGTT 660

A G S T W E V F V P A G L A Y G P R S V 220

backward

GGCGGACCTATTGGCCCAAATGAACTTTAATATTCAAAATTCACCTAATTTTCAGTGAAA 720

← G G P I G P N E T L I F K I H L I S V K 240

AAATCATCTTAAACCTGCATCTTTTGCTGCCGGTACCGTCGACCGCTTCTGCATTAAAG 780

K S S * 243

GGTCAAGAATCAGAGTTAACCGATATCA 808

site (RBS) GGGGA was positioned six bases upstream of the ATG codon. However, the transcription termination signal typically represented by inverted repeat sequence was not found downstream of the stop codon. The 243 amino acids had a calculated molecular weight of 27,300, which is slightly lower than 30,000 estimated by SDS-PAGE. The predicted pI value from the primary structure was 10.02 (Fig. 7), which was consistent with the results in Fig. 2. The amino acid sequence of Mipx, with a highly basic pI value, contained approximately 10% lysines (25 residues) as did the sequences of *L. pneumophila* and *C. trachomatis* homologues. The hydropathy plot and secondary structure of Mipx (Fig. 8), analyzed by using combination of methods of Chou-Fasman (1978) and Garnier-Osguthorpe-Robson (1978), showed that the initial 23 amino acid residues were hydrophobic and a typical α -helix structure was predicted. This could represent a secretory signal, but no typical signal sequence cleavage site was identified using the prediction algorithm of Von Heijne (1983). These analyses also showed that the remainder of the polypeptide was divided into two distinct regions. Residues 50 through 115 were predicted to form an uninterrupted α -helix and the remaining C-terminal domain formed β -sheets and turns, interrupted only by one small segment of α -helix from residues 185 to 197. The extended α -helix of Mipx from residues 50 to 115 could represent an elongated structural projection from the surface of the bacterium.

The nucleotide sequence of the *mipx* gene was very similar to the corresponding sequences of *Legionella micdadei*, *L. pneumophila* and *Chlamydia trachomatis* genes, with an identity of 79%, 74% and 61%, respectively. Among 108 amino acids at the C-terminal region, 38 amino acids exactly matched with those of the human FKBP, which showed a identity of 35% (Fig. 9). Six amino acids out of ten known to be involved in the interaction of human FKBP and FK506 were conserved in the Mipx protein (— in Fig. 9). In addition, three amino acids, Asp-172, Tyr-215 and Phe-232 (corresponding to Asp-32, Tyr-82 and Phe-99 in the

Figure 7. Isoelectric point (pI) of Mipx protein.

The estimated pI value of Mipx protein is 10.02, showing that the Mipx protein is a highly basic protein.

Isoelectric point = 10.02

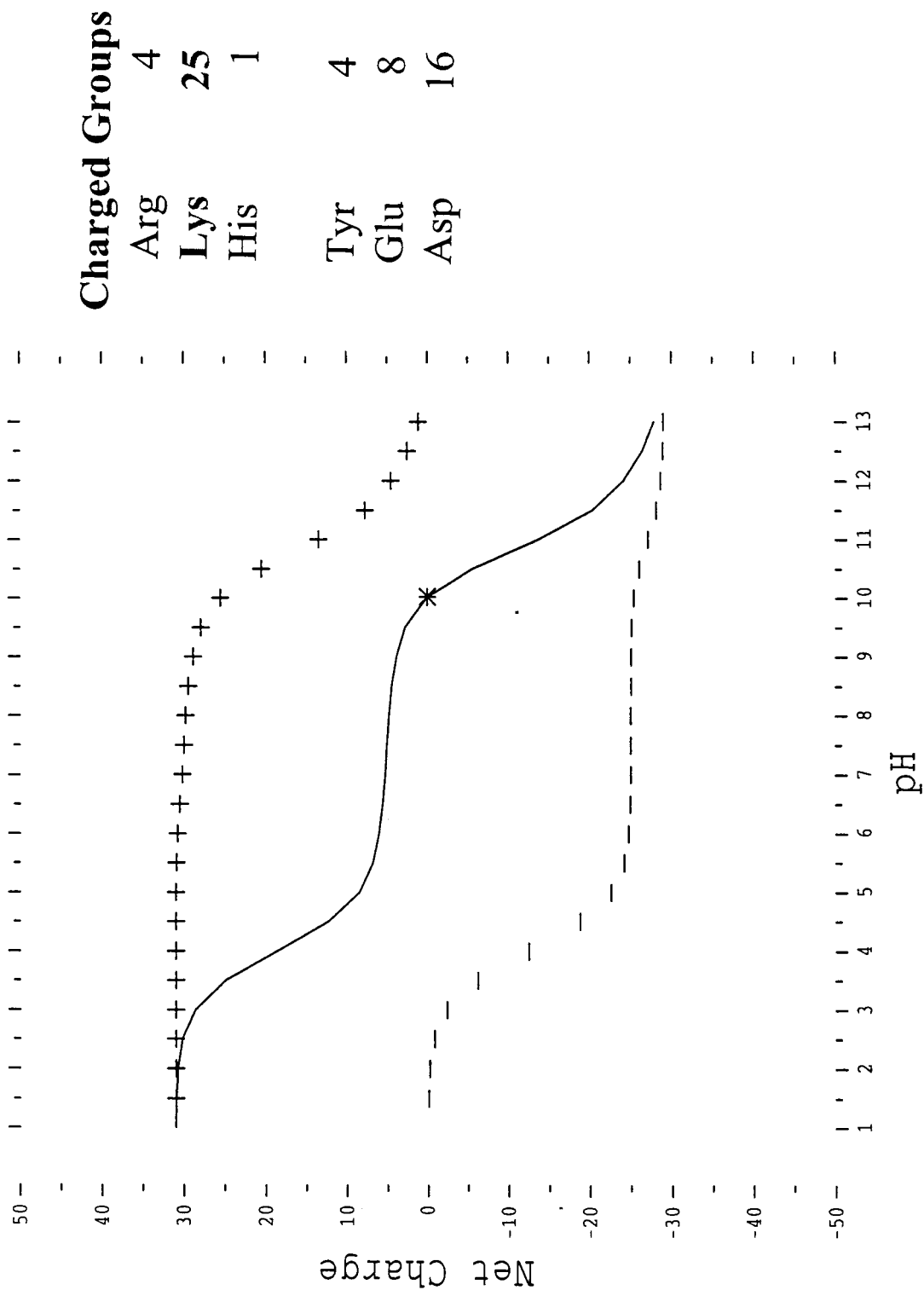
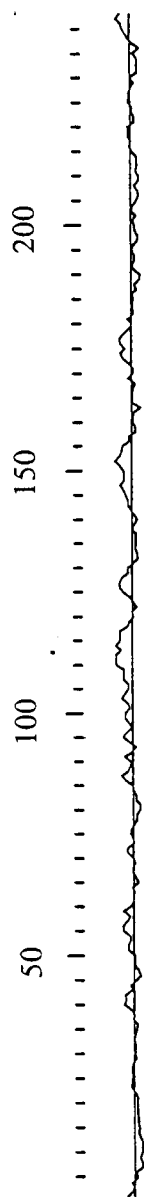


Figure 8. Secondary structure and hydropathy prediction plot of the Mipx protein. Scales at the top and bottom correspond to the polypeptide residue number. The secondary structure prediction at the bottom was made by the method of Chou-Fasman and Garnier-Osguthorpe-Robson.

The initial 23 amino acids were hydrophobic, residues 50-115 were predicted to form an uninterrupted α -helix ([] of GOR) and the remaining C-terminal part formed β -sheets and turns, interrupted only by a short segment (residues 185-197) of α -helix ([] of CF).



hydrophilicity



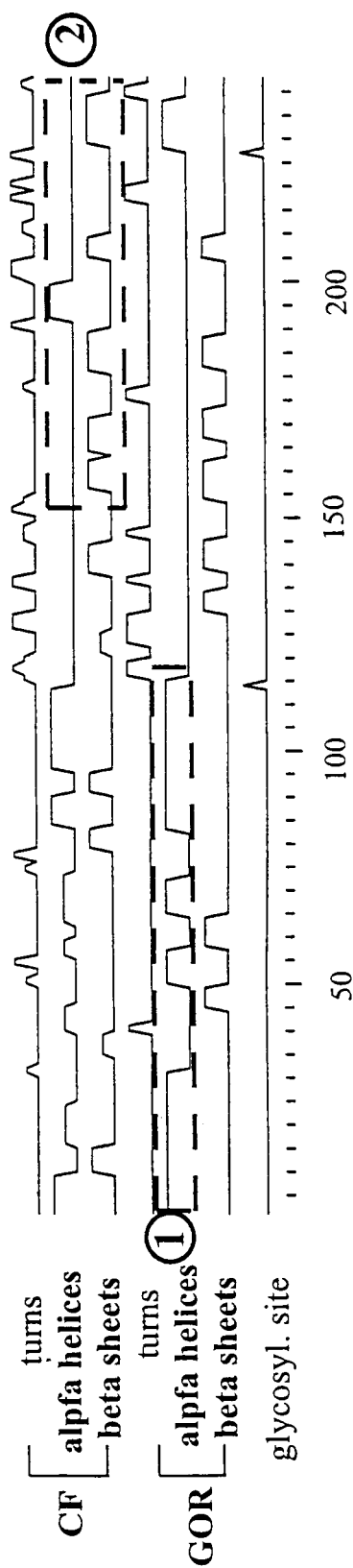
surface prob.



flexibility



antigen index



human FKBP) which play a major role in the PPlase activity and in FK505 binding were well conserved in the Mipx protein.

3. Expression of *mipx* in *E. coli* and peptidyl-prolyl *cis-trans* isomerase (PPlase) activity of recombinant protein

Based on the sequence homology and secondary structure of the C-terminal region, it was reasonable to assume that Mipx might have peptidyl-prolyl *cis-trans* isomerase activity. In order to test this hypothesis, the entire *mipx* gene was first ligated into pET-30b expression vector and inserted to transform BL21 (DE3) strain of *E. coli*. Several attempts at expression of entire *mipx* gene were unsuccessful, however. The *mipx* gene truncated 61 amino acids of N-terminal end was introduced into the *EcoRI/EcoRV* site of the pET-30a vector to be expressed as a fusion protein containing a His-tag stretch sequence (Fig. 10). The overproduction of recombinant Mipx was achieved by induction with 1-mM IPTG (Fig. 11, Lane 3). When *E. coli* carrying pET-30a/*mipx* were extracted with different detergents, the protein was resolved mainly from 6-M Urea fraction, indicating that the overproduced fusion protein was localized in inclusion bodies (Fig. 12, Lane U). Since the recombinant Mipx fusion protein contained His-tag sequence, it was purified with a Ni^{2+} -affinity chromatography column (Fig. 11 Lane 4). A 400-mM imidazole solution was used as the elution buffer and the eluent was dialyzed against 100-mM Tris-HCl (pH 7.4) to remove imidazole prior to concentration by Centricon-10 to appropriate concentrations. The recombinant protein produced by *E. coli* also cross-reacted with an anti-Mip antibody against *Legionella* (Fig. 13B).

The PPlase activity of recombinant truncated Mipx was measured in terms of *cis/trans* isomerization of the substrate synthetic peptide N-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide in a coupled assay with chymotrypsin which hydrolyzes the anilide bond in the *trans* form of the Ala-Pro peptide bond. PPlase helps accelerate

Figure 9. Alignment of amino-acid sequences of FKBP homologues from various sources.

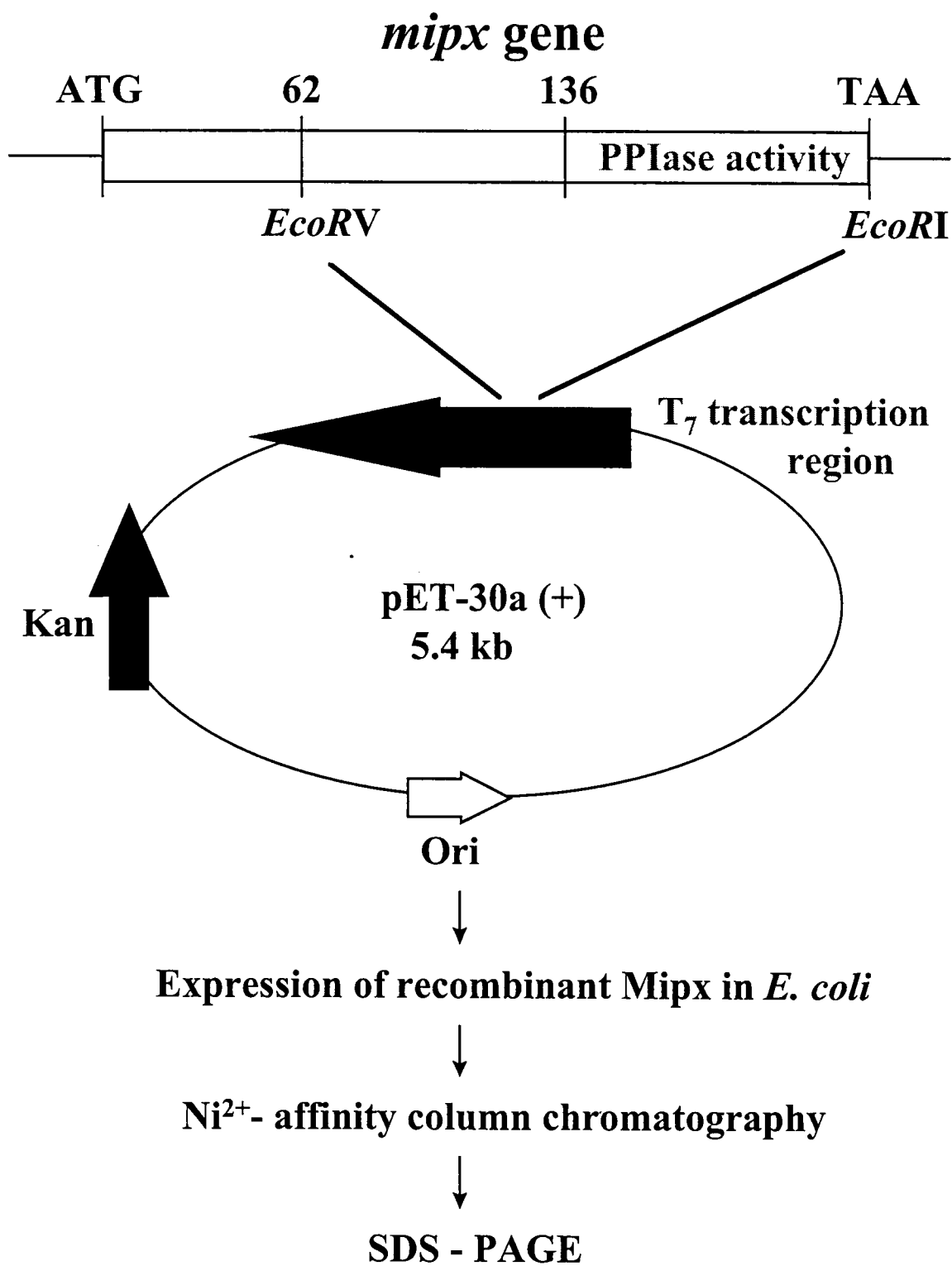
Xb; X-bacteria
Lm; *Legionella micdadei*
Lp; *Legionella pneumophila*
Ct; *Chlamydia trachomatis*
Hf; human FKBP12

Identical amino-acid residues are indicated in bold type and highlighted. Dashes represent gaps for optimal alignments. The underlined amino acids in human FKBP12 are those involved in FK506 binding and PPlase enzymatic activity.

Xb	---	MMRLV	AA	TVIGLAMS	TTMAASAAPD	AATTASSATS	LT	TDMDKLSY	SIGADLGKNL	56
Lm	---	MMRLV	AAA	AMGLAMS	TTIAAIAATD	-ATTSAPGTS	LT	TDTEKLSY	SIGADLGKNF	55
Lp	---	MMKLV	EAA	VMGLAMS	TAMSSTD---	-----ATS	LA	TDKDKLSY	SIGADLGKNF	46
Ct	MKNIL	SWMLM	FA	VALPIVGC	DNGGGSQTS	-TEKSMVEDS	AL	TDNQKLSR	TFGHLLARQL	59
Hf	---	---	---	---	---	---	---	---	---	---
Xb	KKQ	GIDISPA	AMAK	GLQDGI	SGQLLLTEQ	QMKDVLNKFO	KDL	MARRTAD	FNKKAENKT	116
Lm	KKQ	GIEISPA	AMAK	GLQDGM	SGQLLLTDD	QMKDVLNKFO	KDL	MMKRSAE	FNKKAENKS	115
Lp	KNQ	GIDVNPE	AMAK	GMQDAM	SGAQLALTEQ	QMKDVLNKFO	KDL	MAKR TAE	FNKKADENKV	106
Ct	SR	-----TE	DFSLD	LVEVI	KGMQSEIDGQ	SAPLTDTEYE	KQ	MAEVQKAS	FEAKCSENLA	113
Hf	---	---	---	---	---	---	---	---	---	---
Xb	KGDS	FLNQNK	GKD	GVVSLPS	GLQYKVIQKG	DGAKPSK-ED	TVE	VDYTGTL	LDGQVFD TTE	175
Lm	KGEA	FLNENK	SKEG	VVSLPS	GLQYNILERG	DGAKPTK-DD	VVT	VEYTGKL	IDGQVFDSTE	174
Lp	KGEA	FLTENK	NKPG	VVVLPS	GLQYKVINSG	NGVKPGK-SD	TVT	VEYTGRL	IDGTVFDSTE	165
Ct	SAEE	FLKENK	EKAG	VIELEP	NKLHDRVVKE	GTGRVLSGKP	TALL	HYTG SF	IDGKVFD SSE	172
Hf	---	---	---	-----M	GVQVETISPG	DGRTFPKRGQ	TCV	VHYTGML	EDGKKFDS SR	41
Xb	KTGK	PATFKV	S--Q	VIPGWT	EALQLMPAGS	TWEVFPAGL	AYG	PRSVGGP	IGPNETLIFK	233
Lm	KTGK	PATFKV	S--Q	VIPGWT	EALQLMPAGS	TWEVYIPSNL	AYG	PRSVGGP	IGPNETLIFK	232
Lp	KTGK	PATFQV	S--Q	VIPGWT	EALQLMPAGS	TWEIYVPSGL	AYG	PRSVGGP	IGPVETLIFK	223
Ct	KNKEP	--ILL	PLTK	VIPGFS	QGMQCMKEGE	VRVLYIHPDL	AYGT	---AGQ	LPPNSLLIFE	227
Hf	DRNKP	FFK FML	GKQ	EVIRGWE	EGVAQMSVGQ	RAKLTISP DY	AYG	ATGHPGI	IPPHATLVFD	101
Xb	IHLIS	VKKSS*								243
Lm	IHLIS	VKKSDA*								243
Lp	IHLIS	VKKSS*								233
Ct	VKLIE	ANDDNVSVTE*								242
Hf	VELL	KLE*								108

Figure 10. Strategies of expression of recombinant Mipx protein in BL21 of *E. coli*.

The *EcoRI/EcoRV* fragment of *mipx* containing PPlase activity domain was introduced into the multi-cloning site (MCS) of pET-30a expression vector. BL21 (DE3) strain of *E. coli* was transformed with pET-30a/*mipx*. For the induction of recombinant protein synthesis, IPTG was used at 1 mM. Recombinant Mipx was purified by Ni^{2+} -affinity column chromatography with a 400-mM imidazole buffer.



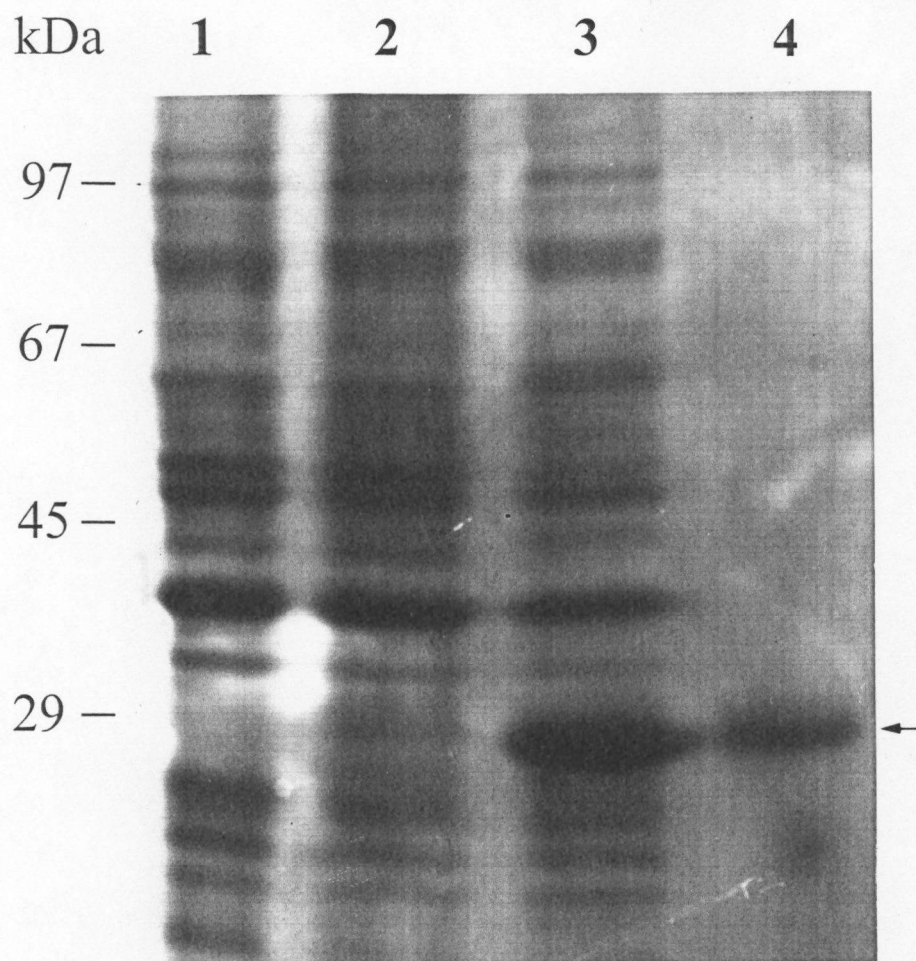


Figure 11. Expression and purification of Mipx protein in *E. coli*.

Commassie-blue-stained SDS gel of whole proteins of *E. coli*

Lane 1. Cells transformed with pET-30a only

Lane 2. Cells transformed with pET-30a/*mipx* without IPTG

Lane 3. Cells transformed with pET-30a/*mipx* with IPTG

Lane 4. Purified Mipx

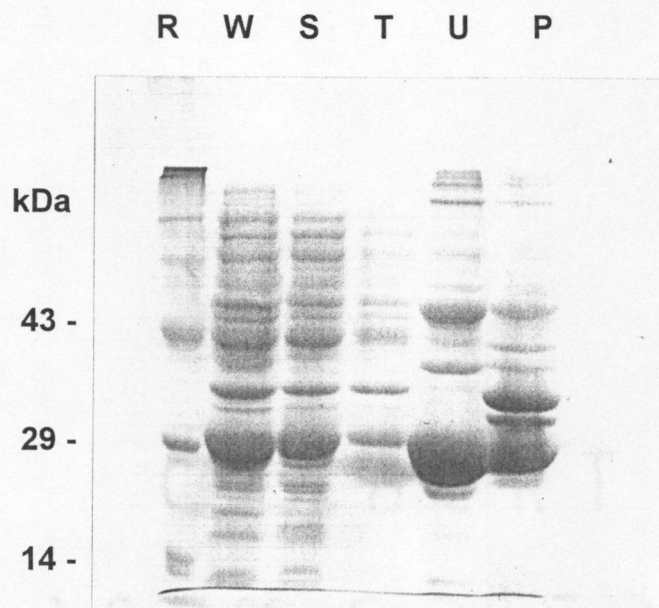


Figure 12. Coomassie-blue-stained SDS-PAGE of detergent-fractions of Mipx produced by *E. coli*.

Lane **W**. Whole-cell proteins

Lane **S**. Soluble fraction of sonicated cells

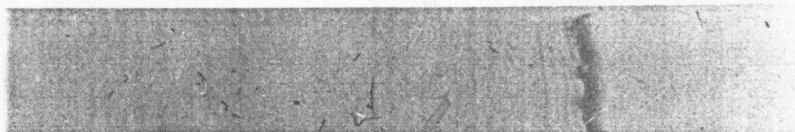
Lane **T**. 2% Triton X 100-soluble fraction of sonicated pellet

Lane **U**. 6-M Urea-soluble fraction of Triton-treated pellet

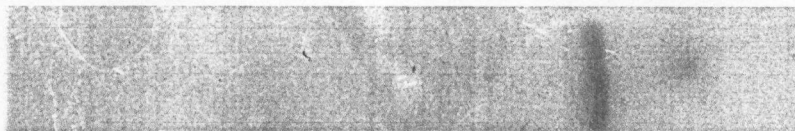
Lane **P**. Final pellet

Figure 13. Coomassie-blue-stained SDS-gel (A) of purified Mipx protein by Ni^{2+} -affinity column chromatography and the corresponding immunoblot (B).

B.



A



100% COTTON

conversion of the *cis* form into the chymotrypsin-cleavable *trans* form. The hydrolysis was measured by at 410_{nm} for 120 sec at 5-sec intervals as seen in Fig. 14. The recombinant Mipx exhibited an PPIase activity (Fig. 14A) with a much greater increase in absorbance than the control without Mipx protein (Fig. 14B). When the effect of drug FK506 was tested on the recombinant Mipx, PPIase activity was strongly inhibited (Fig. 14C). For maximizing inhibition of enzymatic activity, a high concentration (30 μ M) of FK506 was used. These results suggested that the C-terminal region of Mipx had PPIase activity, which could be inhibited by the binding of the immunosuppressant drug FK506.

4. Infectivity assay of X-bacteria with FK506

In order to evaluate the role of Mipx in X-bacterial infection of amoebae, FK506 was used to inhibit PPIase activity of Mipx. The effect of Mipx on infectivity was measured by counting the number of X-bacteria in amoebae at intervals after infection. Isolated 10^9 X-bacteria from xD amoeba were treated with FK506 (30 μ M) or without FK506 as a control for 5 min at room temperature. At the end of incubation, X-bacteria were centrifuged and the pellet was washed with Chalkley's solution. X-Bacteria were further incubated with 0.05% poly-L-lysine for 20 min at 4° C prior to infection into 1×10^4 D amoebae. Amoebae were thoroughly washed with Chalkley's solution to remove remaining X-bacteria 2 hr after phagocytosis. Twenty amoebae were picked up at time intervals and suspended in 2-N NaOH, mounted on a Petroff-Hausser bacteria counter and counted under a phase-contrast microscope. Infectivity of X-bacteria pretreated with FK506 was reduced to one third that of untreated X-bacteria (Fig. 15), suggesting that Mipx contributed to the infectivity of X-bacteria in amoebae, similar to the Mip of *Legionella* and *Chlamydia*.

Figure 14. The peptidyl-prolyl *cis/trans* isomerase (PPIase) enzyme activity of recombinant Mipx protein.

- A.** Recombinant Mipx protein
- B.** Control
- C.** Recombinant Mipx protein with FK-506 present

The PPIase activity of recombinant Mipx protein was assayed by a coupled method with α -chymotrypsin as a protease and synthetic peptide, N-succinyl-Ala-Ala-Pro-Phe-nitroanilide, as a substrate. The absorbance was measured at 410 nm at 5-sec intervals over a period of 120 sec.

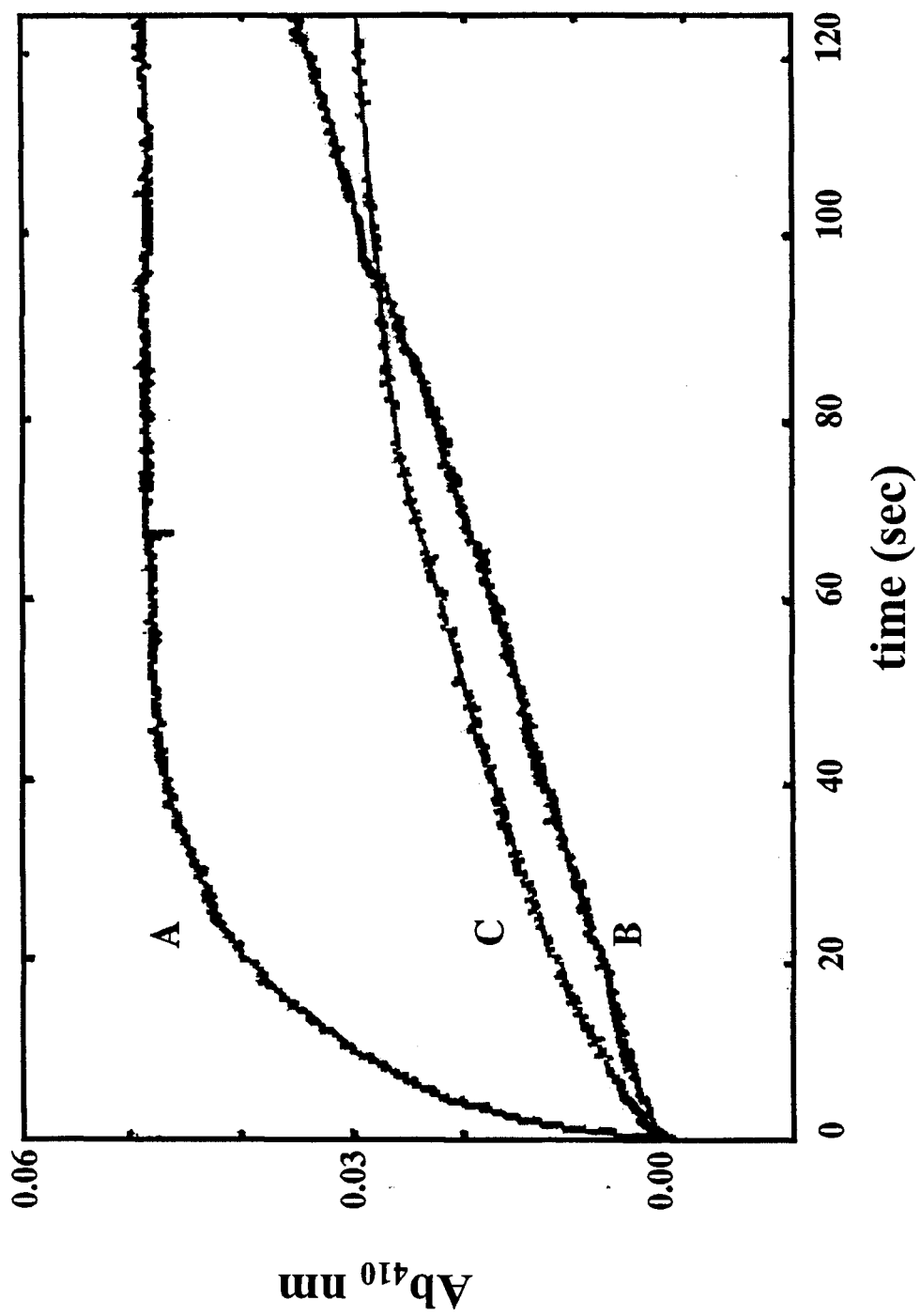
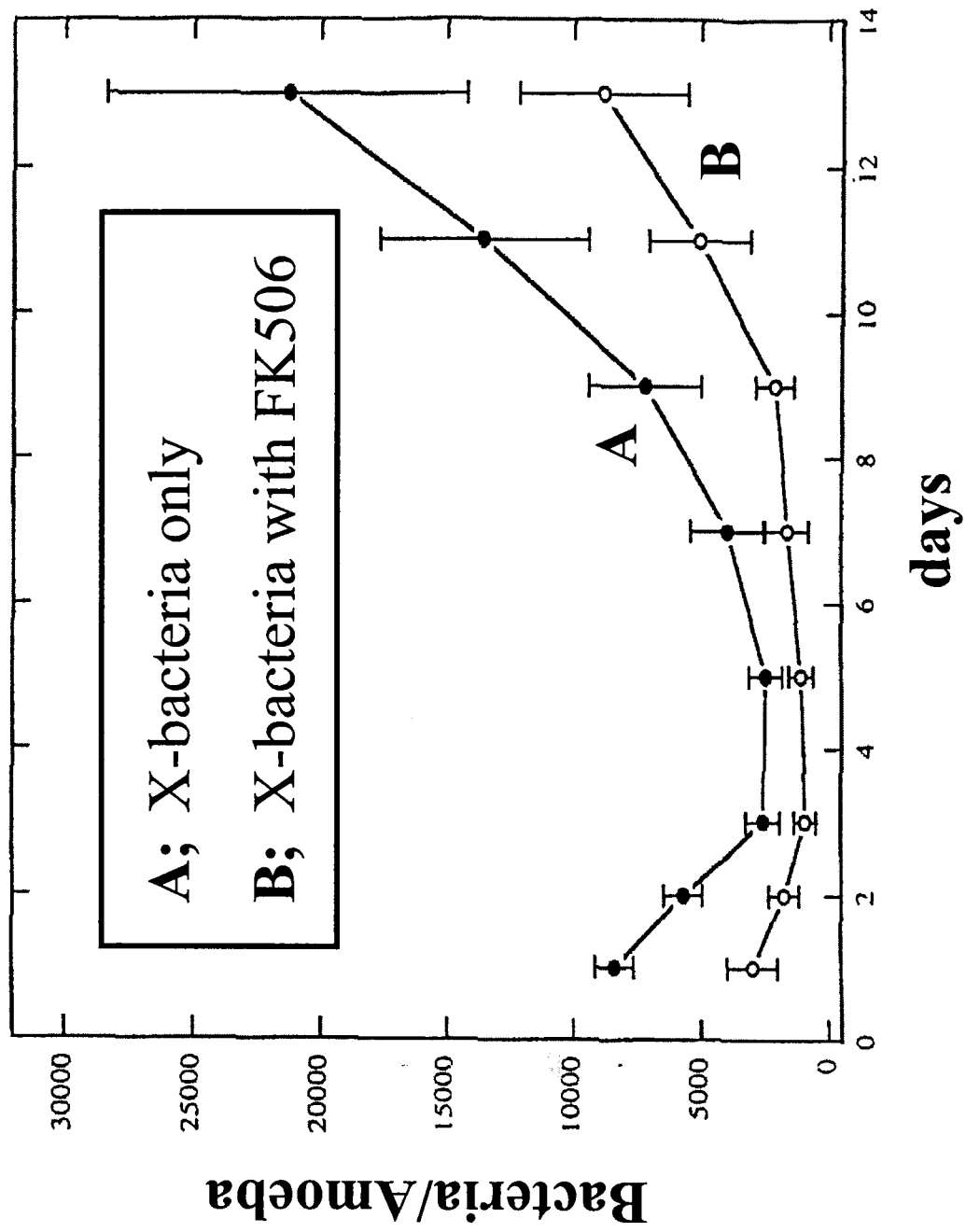


Figure 15. Infectivity assay of X-bacteria in amoebae.

A. X-Bacteria only (control)

B. X-Bacteria pretreated with FK-506

X-Bacteria were experimentally introduced into D amoebae by phagocytosis. The infectivity of X-bacteria was evaluated by counting the number of X-bacteria per amoeba at intervals after infection.



B. Myosin and Its Gene in Amoebae

1. Anti-myosin mAb and the localization of myosin in amoebae

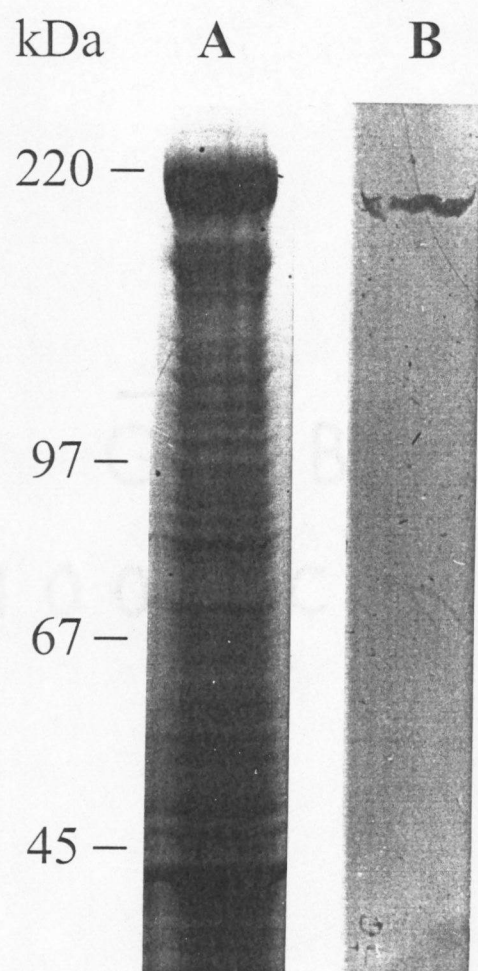
From three fusions of myeloma cells and spleen cells of mice injected with 220/210-kDa protein from D amoebae, many clones of hybridoma cells were obtained. Among them, three clones (CJM50, CJM72 and CJM111) were selected producing mAbs that recognized not only cytoskeletal proteins of amoebae as checked by SDS-PAGE and immunoblotting (Fig. 16) but also nuclear proteins of tissue culture cells such as HeLa and Vero cells (Fig. 17). When amoebae were stained with an FITC-labeled mAb, the antigen was found dispersed throughout the cytoplasm with a high concentration in the ectoplasmic regions of D amoebae (Fig. 18A, a). In xD amoebae, the mAb also recognized proteins present on symbiosomes (Fig. 18B, b) where anti-actin mAb recognized its antigen as well (Fig. 19). That the antigen in xD amoebae was present on symbiosome membranes was confirmed by immunoelectron microscopy using a secondary antibody tagged with horse-radish peroxidase as a probe (Fig. 20). The result suggested that the amoeba's myosin was involved in the maintenance of the structure of symbiosomes together with other cytoskeletal proteins such as spectrin and actin (Choi & Jeon, 1989a, b; Kim & Jeon, 1987) that had been detected on symbiosomal membrane. In addition, the antigen was concentrated in the constricted area, corresponding to division furrow of a dividing amoeba, as checked by immunofluorescence microscopy (Fig. 21), indicating the involvement of amoeba's myosin in cytokinesis as seen in other organisms (De Lozanne & Spudich, 1987; Spudich, 1989).

2. cDNA cloning and characterization of myosin heavy chain gene

Using mAb CJM111 as a probe, the cDNA library of *A. proteus* was immunoscreened and six positive phage clones were obtained. After the phage

Figure 16. Coomassie-blue-stained SDS-gel (A) of cytoskeletal proteins of amoebae and the corresponding immunoblot (B).

In **B**, the anti-myosin monoclonal antibody CJM72 reacted with a 210-kDa polypeptide of amoebae's proteins.



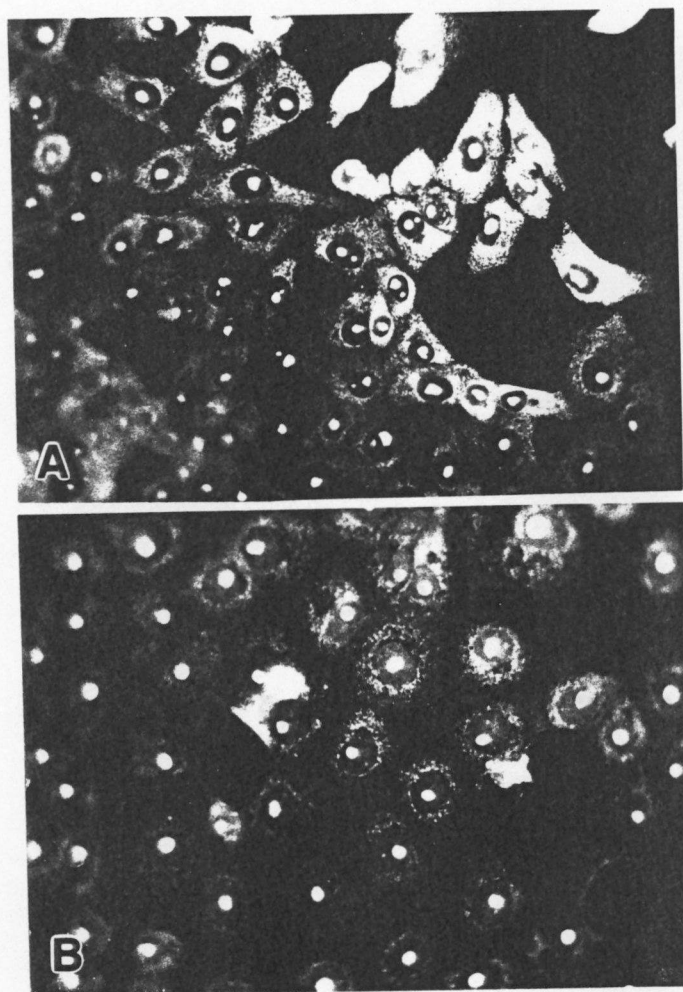


Figure 17. Indirect immunofluorescence micrographs of HeLa (A) and Vero (B) cells stained with the anti-myosin monoclonal antibody CJM111.

In both (A, B) cells, proteins in inside nuclei were recognized by the mAb111.

Figure 18. Phase-contrast (A, B) and indirect immunofluorescence (a, b) micrographs of D (A, a) and xD (B, b) amoebae stained with the CJM111 mAb.

Myosin was found dispersed throughout the cytoplasm with a higher concentration in the ectoplasmic regions of amoebae (a, b). In xD amoebae (b), symbiosomes (arrows) were also stained. Bar = 50 μ m.

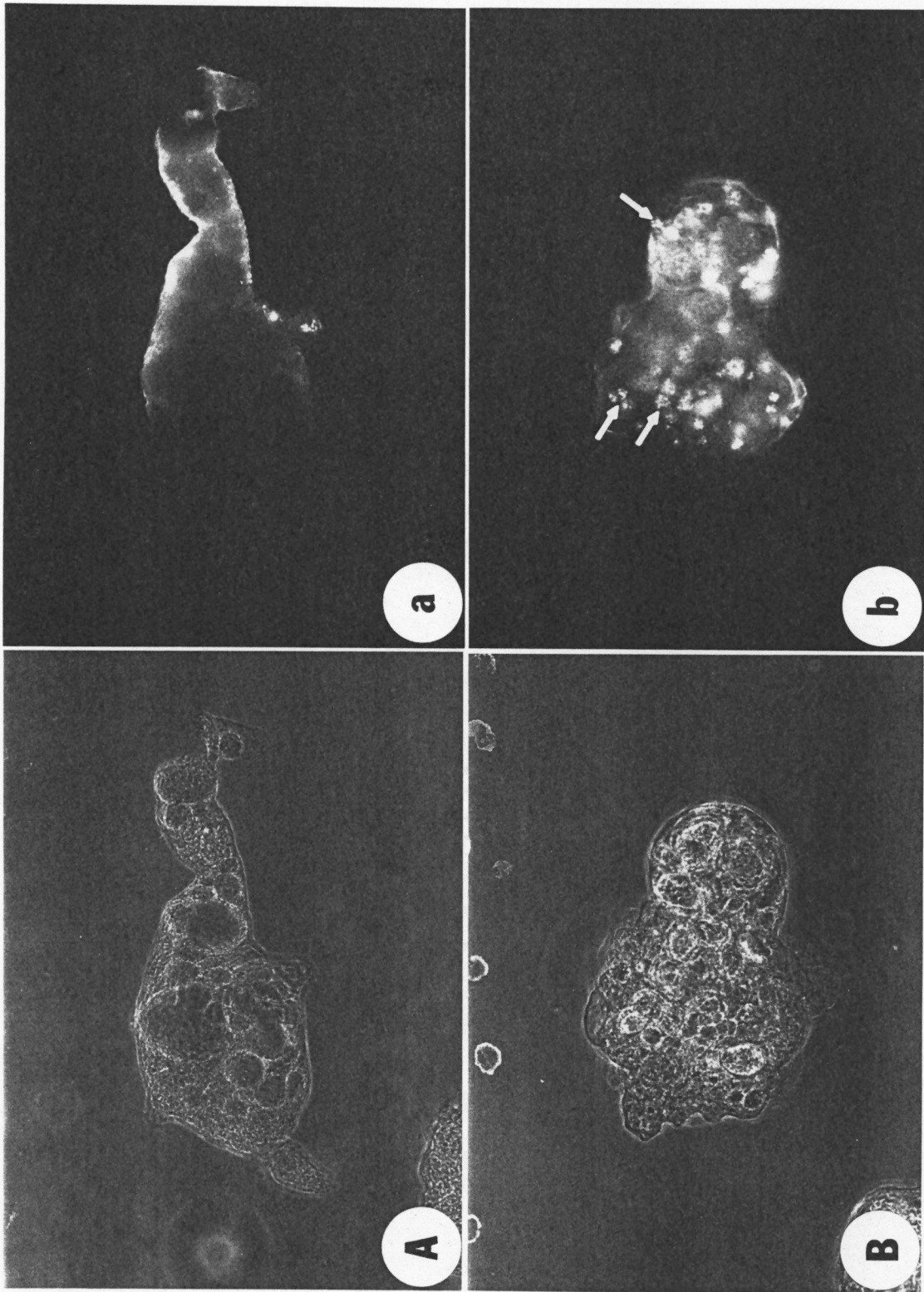


Figure 19. Phase-contrast (A) and indirect immunofluorescence (a) micrographs of an xD amoeba stained with an anti-actin monoclonal antibody KJA43.

In **a**, the mAb recognized the antigen in cytoplasm as well as symbiosomes. Overall pattern of immunofluorescence signal was as same as seen with anti-myosin antibody in Fig. 18.

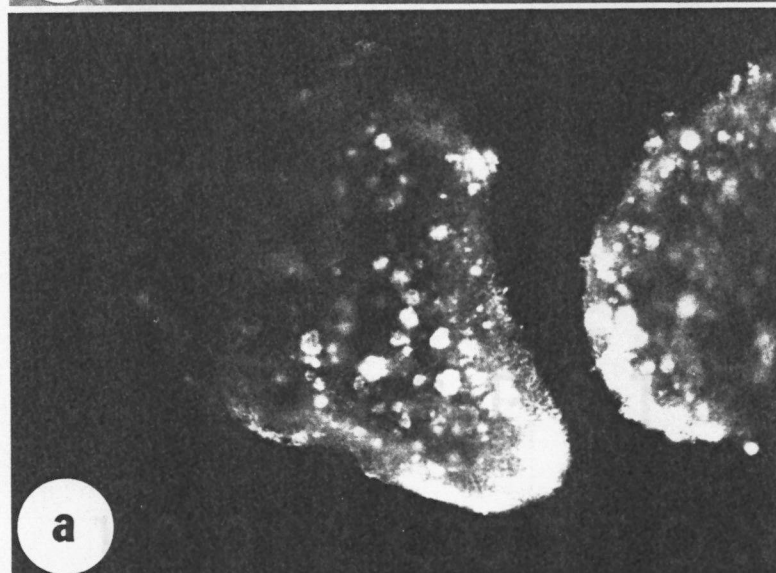
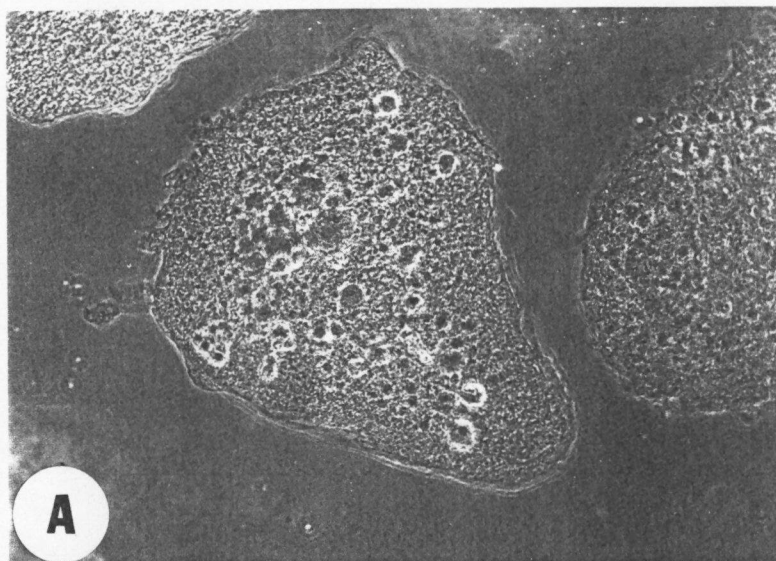
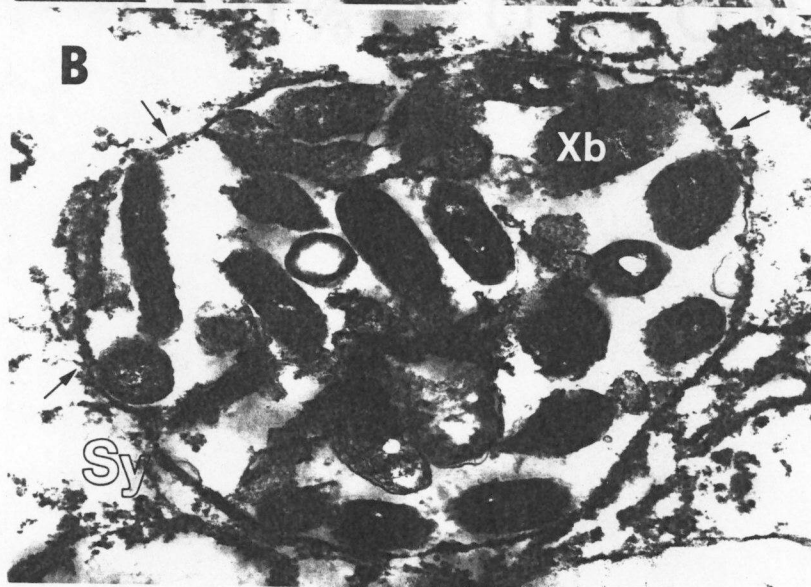


Figure 20. Electron micrographs of xD amoebae to show the presence of an antigen on the symbiosomal membrane.

xD Amoebae were processed without (**A**) or with (**B**) the primary antibody, CJM111, followed by treatment with the secondary antibody conjugated with horse-radish peroxidase. Arrows in **B** indicate reaction products on the symbiosomal membrane. Bar = 1 μm .



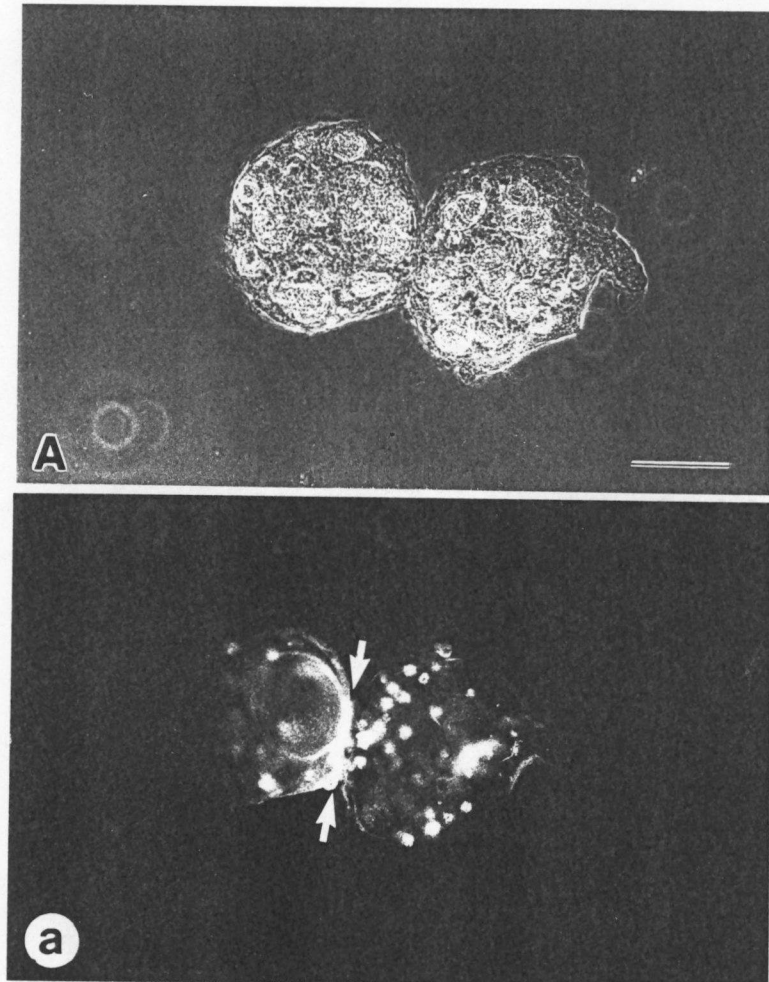


Figure 21. Phase-contrast (A) and indirect immunofluorescence (a) micrographs of a dividing amoeba stained with anti-myosin mAb50.

In **a**, the antigen was concentrated in the constricted region (arrows) corresponding to a division furrow in the dividing amoeba.

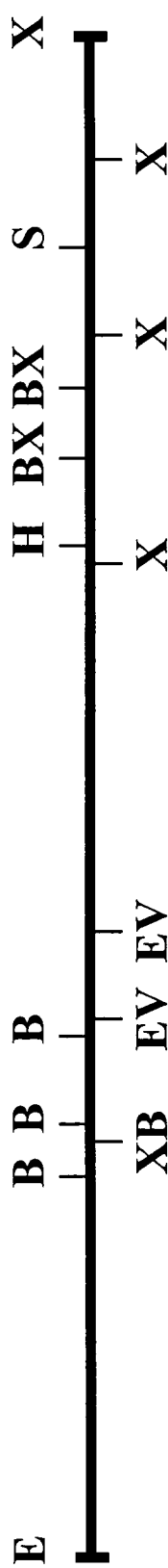
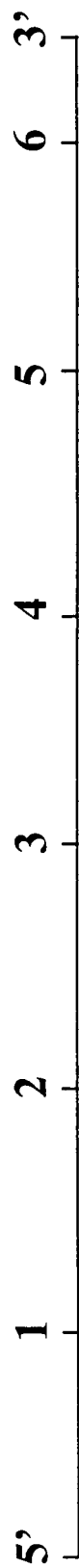
clones were autoexcised into pBSK phagemids, fragmentation patterns of the insert cDNA were compared with several restriction enzymes. Among the clones, a pAMHC20 clone containing the largest cDNA insert was selected, mapped (Fig. 22) and its nucleotides sequenced on both strands (Fig. 23). The cloned pAMHC20 was about 500 bp shorter at its 5'-end as compared to known myosin genes of other cells. Thus, PCR was performed using library vector-specific sequences (forward), 5'-GCCGCTCTAGAACTAGTG-3' and myosin gene specific sequence (reverse), 5'-ACCAGACTCACCAGTGAT-3' as a primer, and the cDNA library of amoebae as a template. A 605 bp PCR product was cloned into pBSK⁻ (pAMHC5) and its nucleotides were sequenced to complete the sequence of the amoeba's gene. The cloned myosin cDNA contained 6,499 bp including a poly-A tail and it encoded a protein of 2,138 amino acids (Fig. 23), with an estimated M_r of 235 kDa (close to M_r of myosin II), slightly larger than 210 kDa determined by SDS-PAGE (Fig. 16).

By searching nucleotide and amino-acid sequences in databases, it was found that the deduced amino-acid sequence of the presumptive globular heads of amoeba's myosin showed a high degree of identity with that of myosin genes that had been characterized in many different organisms including human (Eller et al. 1989; Warrick & Spudich, 1987). When amino acid sequences of various myosin II heads were aligned, regions with a high similarity were found scattered across the entire globular head and their positions relative to the primary sequence of the myosin head were highly conserved in all myosins compared. The conservation of sequence topology is shown in Fig. 24 with the alignment of amino acids of myosin heavy chain of *Amoeba* and *Dictyostelium*. The alignment produced a single strong diagonal spanning through the entire globular head region. Among regions showing a high degree of homology, the ATP-binding site (residue 161 - 196) of amoeba's myosin contained the G-E-S-G-A-G-K-T sequence that matched the sequence of all myosins examined (* in Fig. 25A). In addition, residues 649-671 contained the P-H-

Figure 22. Restriction enzyme maps and a schematic structure of the myosin heavy chain (MHC) cDNA of *Amoeba proteus*.

The mapped restriction sites are *Bam*HI (B), *Bst*XI (BX), *Eco*RI (E), *Eco*RV (EV), *Hind*III (H), *Sac*I (S), *Xba*I (XB), and *Xho*I (X). pAMHC20 is an original cDNA clone of 6,050-bp and pAMHC5 is a clone of 566 bp acquired from PCR. DNA sequencing was performed using synthetic oligonucleotides from an automatic sequencer or by making deletion ladders with *Exo*III/mung bean nuclease. The positions of ATP and Actin-binding site of the globular head region are indicated with arrows in schematic structure of MHC. Black boxes represent *Bam*HI fragments used as probes for Northern blot hybridization.

MHC cDNA map

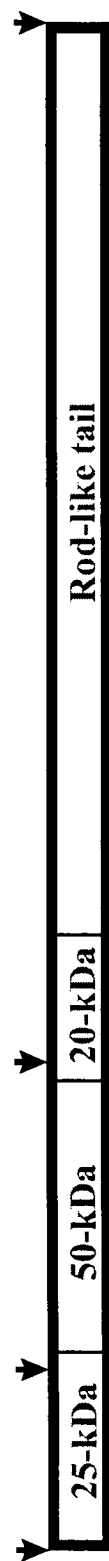


pAMHC20

74



ATG ATP-binding Actin-binding



probe



Figure 23. Complete nucleotide and amino-acid sequences of cDNA encoding 210-kDa amoeba myosin.

The start (**ATG**) and termination (**TGA**) codons are in bold letters, and the putative polyadenylation signal (**AATAAA**) is underlined and bold-typed. The lysine (**K**) residue (840) corresponding to proline of a landmark between the head and tail in other myosins is underlined and highlighted. An unusual aromatic amino-acid stretch just before the lysine residue (840) is also underlined. The cDNA has 6,499 nucleotides and encodes a protein of 2,138 amino acids.

3361 AACGATCAGACTGAAGATGAGAACCAACAAAAAGAGACTCTTTCCACCAGCTCAAGAAAGTTGGTGATGATTTGGCAGACGTGAGATCTCACATTGATGATGAG
N D Q' T E D E N N K K K T L S N Q L K K V G D D L A D V R S H I D D E 1155
3466 CACAATCAAAAGTTACGACTCACTAATGAAATACTCGTCTTGAGGCTGCAATCGATGACCTGAAGCGTCAGTTGGATGAGACCAAGGAAAGATTTCGAAGTTG
H N Q K L R L T N E N T R L E A A I D D L K R Q L D E T K G K I S K L 1190
3571 GAGAAGGAGAAGCAACAGTTGAGAGATGTACGGCTCAGTTTGAAGATGCTGAGAACAGTTTCTCAATTGACCAAGCAACCTCAAATC
E K E K Q Q L Q K H L E D V T A Q F E D A E N K F S Q L T K T N L K L 1225
3676 AAAGCTGATTTGGATGAATTGAGGATAACAGGGAAGGTGGTACCAGGCATTCAGAAATTGAAGAAAATGGTTGCCAAGTTGGAGTCTGACAAGAAAATGAAG
K A D L D E L Q D N R E G G D Q A F Q K L K K L V A K L E S D K K M K 1260
3781 GAAAAAGAAATATGAGGACGAGAGATCTCAAGAACAACTGGATGCTCAGAAAAAGTTGCTCAAGCTGAATTGGATGGTCTGAAGAAATGCACTGGAAAGAAATG
E K E Y E D E R D L K N K L D A Q K K L S Q A E L D G L K N A L E E M 1295
3886 GCCAAGATAGGTCCAGAGAAGAGAAGAACAGGAAAGACTTGGAAAAACAGATTACGCGAACTGGAGGATCAGGCTGAAGATGGCCAAAGCTGCACGATCCAATCTA
A K N R S R E E K N R K D L E N R L R E L E D Q A E D G Q A A R S N L 1330
3991 GAGAAGAAATTCAGAGGATTTGAGGATAATTTGGAAGACCATCAAAGTCAGGTGGATGAAGTTCCAGGATGATGTGAACGTGCTCAGTGCTGCAAAAAAGAAATC
E K K F R G F E D N L E D H Q S Q V D E V Q D D V N V L S A A K K K L 1365
4096 GAGTCTGAATTTGAAGACTTGAAGAGATCACTGGATAACGAAGCTGAAGGTAGGAAGTTGCAGAAGAGAAGATGAAGTGTGGATACTGAACTCCATGAACTT
E S E L E A L K R S L D N E A E G R K V A E E K M K V L D T E L H E L 1400
4201 CAGCTTGCACTCTCAATGCGGAGAATAAAAAATACTGGTTTGGTTTCAAGATGTGAAGAAAGTACAGGATGAGGTAGAAGACCTGAATGAGCAGTATGAAAATGCA
Q L A L S N A E N K N T G L V R N V K K V Q D E V E D L N E Q Y E N A 1435
4306 AGTAAGGATTTATCCAAGTTGGACAAGGAAAACAAAAAGACAGAGCGGAACTGAAAGAAATGAGGAGACATGTTCCAGGAGTCTCAGAGTTCACTTGATGCTGGT
S K E L S K L D K G N K K T E A E L K E L R R H V Q E S Q S S L D A G 1470
4411 GAATTGAAGTTACGACATACTCAAGATGAATCGATGAATTACATCAACCACTTGAAGATTTGGAAGCCAAGTCCAGTTTCGCTGGAAGGAGTAAGAAGCACTC
E L K L R H T Q D E L D L H Q L E D L E A K S S L E R S S L E R S N L 1505
4516 CAACITCAAGTCGATGATTTGGAAGATACTCAGGAGGAGAACTGGCTGCTAGGACAAAGGCAGAAAGATTGGTTAAGGATCTTGAGGCGGATTGGCAGAGCTT
Q L Q V D D L E D T H E E E L A A R T K A E R L V K D L E A D L A E L 1540
4621 CAGGAAACAGATGAGAACTGAGCCGTTGATGCAAGCTGAAAGGCTTGAAGAGTTAGAGGTTGAAGTTGAAAAGGATGCACTAGGCAATCT
Q E T R V E S E P L M Q A E K A L K S L E V E L V D L K K D A D R Q S 1575
4726 CAAGCCTTTGCTAAGGTTGAGAACGAGCGAAGGTCGCACTGAGGGAATATGAGGACCTTCAAGCTCAATTGGATGAAACTTCCAAGAACTCGGCAATGCTGAT
Q A F A K E N E R R S A L R E Y E D L Q A Q L D E T S K N L A N A D 1610
4831 AGAGCGAAAAAGAACTCAACACTGATCTGGATGAACAATTGTCCAACTAGAAAAAGGCTCTAATGCTCAAAAGAGTCTGGAAAAAGATGAAGAAAGCAGAG
R A K K K L N T D L D E Q L S K L E K A S N A Q K S L E K R L K K A E 1645
4936 AAGGATCTGCGAGCTGCCAAAGCTGCTTCTGCTCGTGGTGGTGGTTCCTGATGAAGAACTGAGAAGGGCAGAGGCTGAACCTTGACGACTTCGGGATGAT
K D L A A A K A A A R A G G G V S D E E L R R A Q A E L A A L R D D 1680
5041 GCCGACAGAGATAAGATCCAACAAGCTCAGGCGAGAAAAAGAGTGAAGAAATCTTCAAGCGGAGATAGAAGATCTGAAGGAGATGCTCGAGGATGAAAAGACCAGC
A D R E R S N K L T A E K R V K N L Q A E I E D L K E M L E D E K T S 1715
5146 AAGGAGGCTTGAACAGGAACAAGCTCTCTGGAACAAGAGTTGGAAGAAATGAGAGAGCAATTAGAAGCTGAAGAGGAGGCTTGAATTAATTGGAAGAAATC
K E A L N R N N K S L E Q E L E E L R E Q L E A E E E A L N Y L E E I 1750
5251 AAACACAAGAAAGATTTGGAGATCAACGAAGCTCAGGAAACAGCTGGATCCGAATCAGAAGCACGTGATAAATTTGAACAAATGAAGAAGAGCTGGAGAGGAT
K H K K D L E I N E L R K Q L D A E S E A R D K F E Q L K N E L E R D 1785
5356 GTTGCTGATGCCAAACCAACTTGAAGCCGAAAAAGAAATCCAGAACAGATGCAGAGAGGGAGGCCAAGAAGGCAGAAAGCCAGTATGATGAATTGAAACACCGT
V A D A K H N L E A E K K S R T D A E R E A K K A E A Q Y D E L K H R 1820
5461 TCGGAGGCTTCCGACAGGGGCAAGATAAATTGGAAAAAGAACGAAAGAGAGCTCTCAAGAACTCAGACTCCTTCTGTAAGAAAGTGAAGCGATCGAAGCCGAG
S E A S D R G K D K L E K E R K R A L K E L R L L R E K V E A I E A E 1855
5566 AAGGACGAACAGGAACGCTGCTGCTTGGAAATTCAGGAGGAGGTGATGCACTCACCAGAGCTCTTGATCTCGAACATAAGGCCCGTGTGCTCAGGAGAAGATT
K D E Q E R L A W K F Q E E V D A L T E A L D L E H K A R V A H E K I 1890
5671 GCCAAGCAATTACGTGTTTCAAGAGACTTTAAGGAGACTGCGGAAGATGCCACTCGTGGAAATCTCGTGGCGATCAGTTGACAGCGAGTTGACAGGCGGAG
A K Q L R V Q L E D F K E T A E D A T R G K S R A D Q L T S E L Q A E 1925
5776 ATCAGGATCTAAAGGATCAACTTGTATGAGGAGGAGAACGCAATCGTGAACCTTGCCAACTTCAAAATTAACAACAAGAGTGCCATTGCGGACTTGAAGAAGGCC
I E D L K D Q L D E E E R N R E L A N F K I N N K S A I A D L K K A 1960
5881 CTGGACAGGGAATTTCTGCCAGGGAAGCCCTCGAGGAGGCTAAACGCCAATTGGAAGAGGCAACAATGAACTCAGGATCAACTTGACGAGGAAAGAGTTTCA
L D R E I S A R E A L E E A K R Q L E R D N N E L R D Q L D E E R V S 1995
5986 CTGGAATTTCTGAAGAGGCGCCAGGAAGAGCTTTCAGAGATTGGAAGACCAATGCTCGTCTCAATGCACTCAATGCTTCAATTGGCAAACTTGAAGAAGGCC
R G N S E R A A R K S F A E L E D T N A R L N A L N A S I G K L E K A 2030
6091 AAGCGAAGAGCCGAAGCCGATTCGTGCAAGCAAGAAACAGCTGGCCGACTTGCAGAAAGAGGAGCCACCGAAGATTCTCTTCGTGCACTGATGGAGGCTGAG
K R R A E A D Y R A S K K Q L A D L Q K K E A T E D S L R A Q L E A E 2065
6196 GTGAGACGCTCAAGTCTCGTCTGGTTGACGAACAGGATCGTGTCTGATGCGCAATCTGATCGCAGAAGAGCCGAGGTGGAGATTAATAAGCTCAGGGATGAG
V R R L K S R L V D E Q D R A A D A E S D R R R A E V E I N K L R D E 2100
6301 GTTCGCGTTTGTCTGATGAATTGGAACGTGCGAAGGCCGAGCCAGACAGGCTCGGAAGACAAACAAGAACTTGAAGAGCGTGACAGAGCTGTAGCTGCGCTG
V R V L S D E L E R A K A E A R Q A S E D K Q E L E E R A R A V A G L 2135
6406 TGGGATCACTGATCGTCTTCCAGTGACCGTTCTTAAAGCCAAATAAGCGTTTCTTAAATCAAAAAA
W D H * 2138

Figure 24. Dot-matrix comparison of amino-acid sequence of myosin heavy chains of *Amoeba* and *Dictyostelium*.

Amino acids of the myosin heavy chains were compared by using the mutational index, which counted exact matches and conservative substitutions. The window size was 25 with a stringency of 20.

Dictyostelium

Amoeba proteus

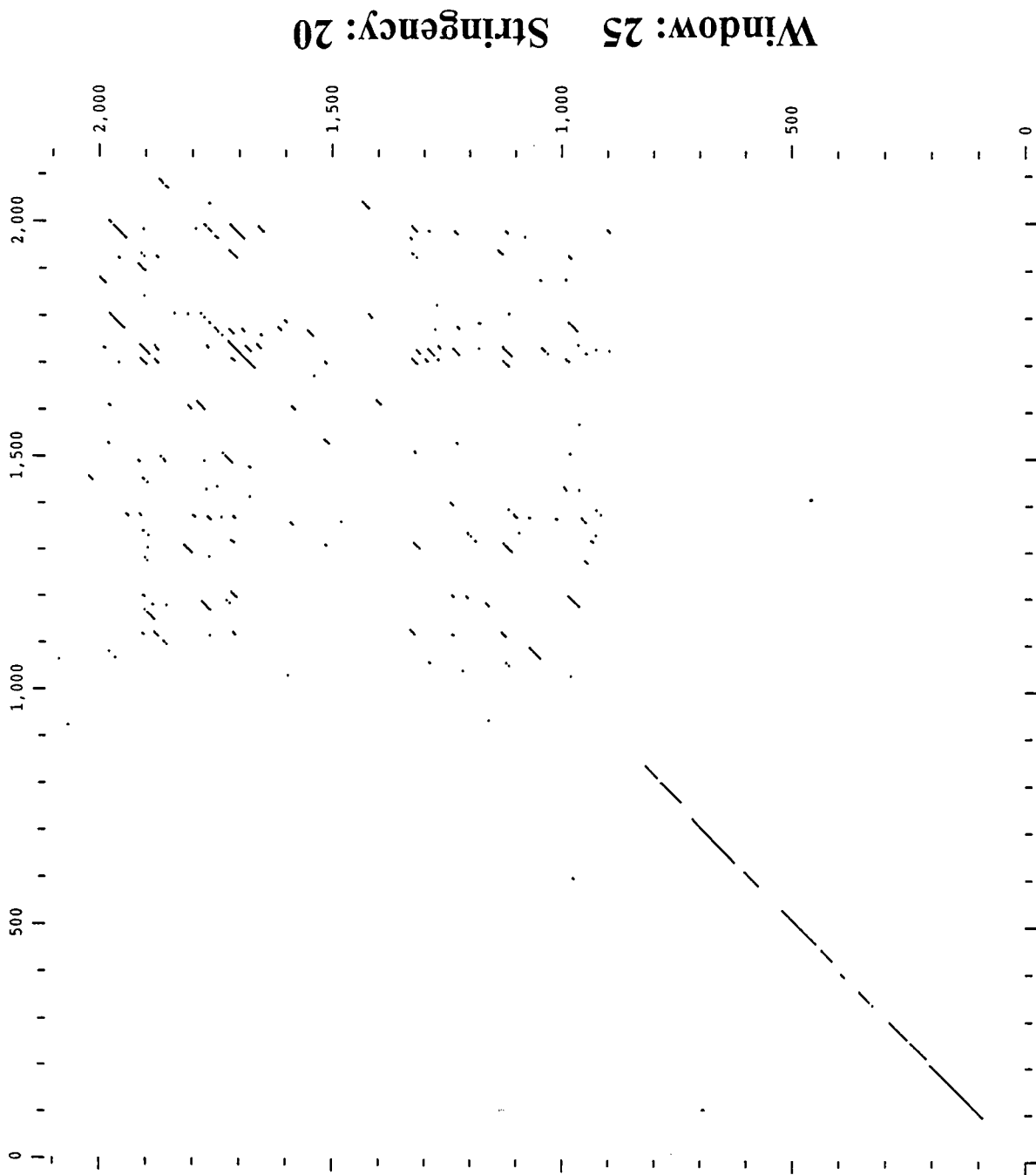


Figure 25. Amino-acid sequences of ATP-binding (A) and actin-binding (B) sites of the globular head of myosin heavy chains (MHC) from various sources.

The bold-typed and highlighted letters represent sequences that contain the same amino acids. Asterisks indicate specific sequence motif appearing on ATP-binding and actin-binding sites.

A. ATP-binding site

<i>Amoeba proteus</i>	DYAYRSLRFN	NEDQSILITG	ESGAGKTENT	KKVIQY
<i>Acanthamoeba typeII</i>	DAAYRAMLNT	RQNQSMILITG	ESGAGKTENT	KKVIQY
<i>Dictyostellium</i>	DVAYRSMDD	RQNQSLLITG	ESGAGKTENT	KKVIQY
Yeast	EEAYENLLSE	GKDDQSILVTG	ESGAGKTENT	KKILQY
Chicken	DNAYQFMLTD	RENQSILITG	ESGAGKTVNT	KRVIQY
Rat	DNAYQFMLTD	RENQSILITG	ESGAGKTVNT	KRVIQY
Rabbit	DNAYQFMLTD	RENQSILITG	ESGAGKTVNT	KRVIQY

B. Actin-binding site

<i>Amoeba proteus</i>	LDDLKLTQK	THPHFIRCII	PNH
<i>Acanthamoeba typeII</i>	LAHLMMLSS	TAPHFIRCII	PNL
<i>Dictyostellium</i>	LASLMATLET	TLPHFVRCII	PNN
Yeast	I.TLLNQLAS	THPHFVRCII	PNN
Nematode	LNNLMTMLNK	THPHFVRCII	PNN
Chicken	LNKLMSNLS	THPHFVRCII	PNE
Rat	LNKLMSNLS	THPHFVRCII	PNE
Rabbit	LNKLMTNLS	THPHFVRCII	PNE

F-X-R-C-X-I-P-N string that was known to be a part of the actin-binding motif (* in Fig. 25B). In all other type II myosins examined, a proline residue was conserved near the S1-S2 junction and it was known to mark the boundary between the head and tail regions. In those myosins, a string of several unusual aromatic amino acids was always found before the proline residue. In the amoeba myosin, there was a stretch of similar aromatic amino acids, WKSWPW, but the proline was replaced with lysine (residue 840 in Fig. 23). The significance of the unique deviation is not clear.

In contrast to the high degree of sequence homology in amino acids of the globular head regions, there was no significant sequence conservation in the rod-like tail region between amoeba's and other organisms' myosin II. Secondary structure predictive methods showed that the tail region of amoeba myosin had a uniform α -helical coiled-coil configuration (Fig. 26). In the amoeba myosin, no other prolines were found in the tail domain as in *Dictyostelium* myosin (Warrick, et al. 1986) and a non- α -helical sequence at the end of the tail was not observed as in *Acanthamoeba* and nematode myosin (Bains & Korn, 1990; Karn et al. 1983). Thus, the MHC gene from the large, free-living amoebae had typical characteristics of myosin II with conserved amino-acid sequences in the head region and α -helical coiled-coil structure in the tail region as in myosins of other organisms.

3. Isoforms of myosin

Since most non-muscle cells including *Dictyostelium* (Jung et al. 1983) and *Acanthamoeba* (Horowitz & Hammer, 1990; Jung et al. 1987) were known to have myosin isoforms, it was checked by Northern blot analysis to see if any subtypes of myosin were present in amoebae using a 1.35-kb DNA fragment containing the ATP-binding site or a 0.4-kb fragment with actin-binding site as a probe. The probes were labeled with ^{32}P -CTP by random-primed synthesis and were hybridized to total

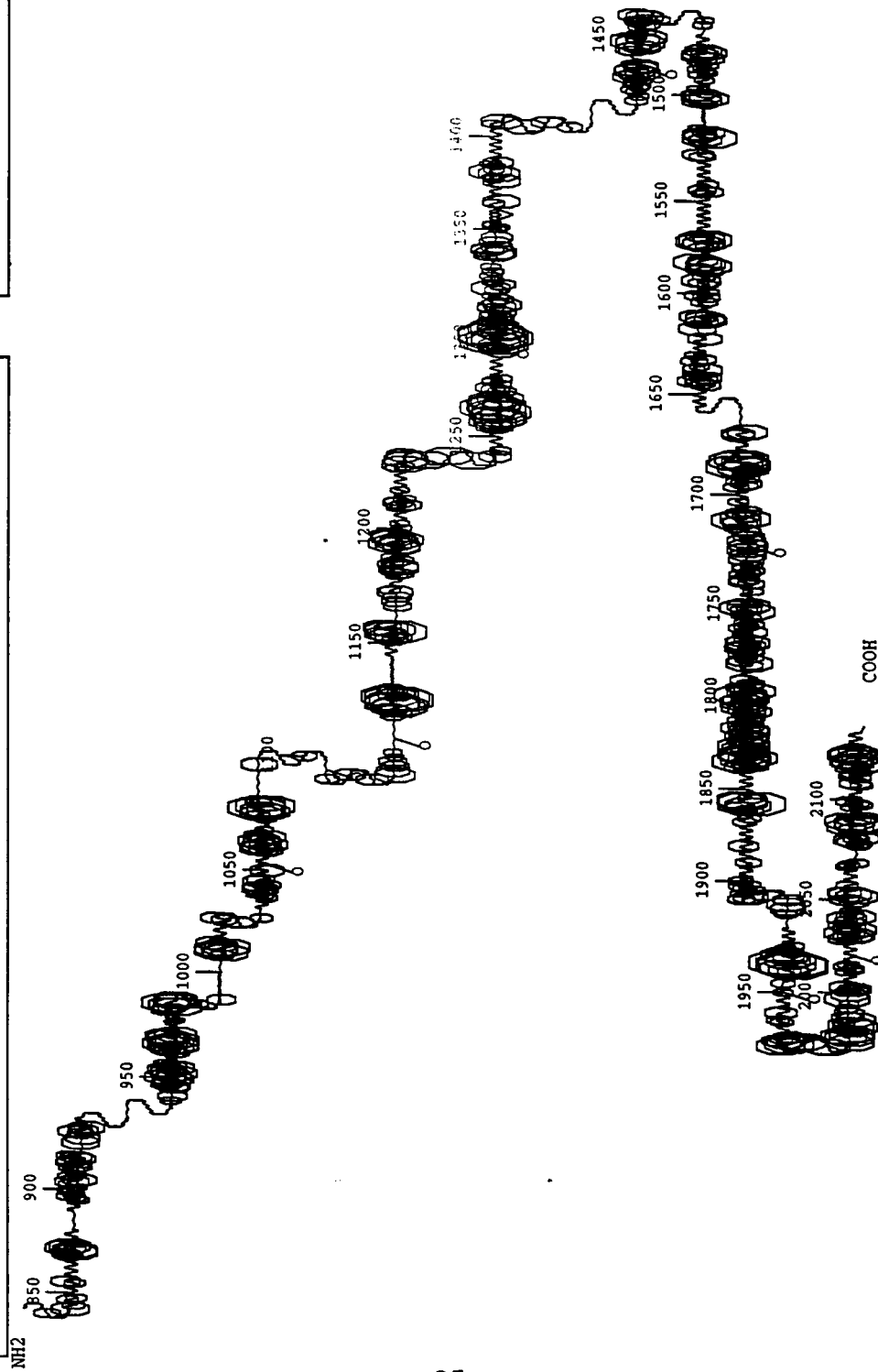
RNA of amoebae under high stringency conditions. Three positive bands with different densities were detected ranging from 4 to 7 kb (Fig. 27). This result indicated that amoebae had at least three different myosin isoforms containing closely related DNA sequences in the globular head region and that the amount of isoforms produced was different. Roles of the amoeba's myosin isoforms in cellular activities are not clear, but the functions of myosin isoforms have been studied in other cells, such as *Acanthamoebae* in connection with the involvement of myosin IC in generation forces to control contractile vacuoles (Doberstein et al. 1993).

**Figure 26. The secondary structure of amoebae myosin's tail region
(residues 841-2138) predicted by the method of Garnier et al. (1978).**

The configuration shows a uniform α -helical coiled-coil structure, indicating that the myosin is a true myosin type-II.

Coiled-coil structure of myosin tail domain

Type II



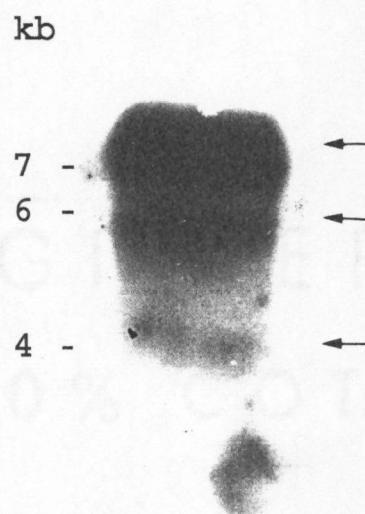


Figure 27. A Northern blot of total RNAs of amoebae probed with a 0.4-kb *Bam*HI fragment.

Arrows point to three bands of 4- to 7-kb RNAs possibly representing three isoforms of amoeba's myosin.

CHAPTER IV

DISCUSSION

A. Mip-like protein in X-bacteria

It has been shown that X-bacteria have a *mip*-like gene encoding a Mipx protein of 243 amino acids (Fig. 6). The molecular weight, isoelectric point and secondary structure based on amino acid composition are very similar to those of *Legionella*. Amino acids at the N-terminal regions (residues 1-135) that are known to be species-specific have a high degree of identity of 79% and 75% with *Legionella micdadei* and *Legionella pneumophila*, respectively (Fig. 9). Considering the high sequence similarity of the C-terminal region of Mipx to the FKBP family, it is not surprising that X-bacterial Mipx protein showed a PPlase activity and FK506-binding properties (Fig. 13).

As far as the PPlase activity is concerned, immunophilins have been characterized extensively (Fruman et al., 1994). Two protein superfamilies of immunophilins can easily be distinguished by their binding properties to immunosuppressant drugs: Cyclophilins (CyPs) are able to bind cyclosporin A (CsA) whereas FK506-binding proteins (FKBPs) bind FK506 and rapamycin. Even though the two superfamilies do not share sequence or structural homology, all CyPs and FKBPs identified to date exhibit a PPlase activity. The action of immunophilin-drug complex on T-cell inactivation has been studied as related to human welfare. Recently a third class of proteins, termed as parvulin, has been described (Rahfeld et al., 1994a; 1994b) and the PPlase enzymatic activity is not inhibited by cyclosporin A or FK506.

Immunophilins are housekeeping proteins in diverse species and organelles with a high concentration up to 1% of the total proteins and strikingly conserved throughout evolution, suggesting that these proteins mediate important cellular functions.

Several recent lines of evidence, in fact, have shown that immunophilins are involved in critical and basic cellular functions including folding and maturation of proteins, membrane channelling, and protein trafficking (Fischer and Schmid, 1990). The *Drosophila ninaA* gene encodes a Cyps homolog with PPlase and its gene product appears to be produced only in retinal photoreceptor cells. Mutations of *ninaA* gene are associated with a decrease of rhodopsin, leading eventually to deficient visual transduction. Thus, NinaA seems to be involved in the folding of this retinal pigment *in vivo* (Stamnes et al., 1991). Folding processes of proteins have been studied by treatment of CsA such as chaperone activity of carbonic anhydrase (Freskgard et al., 1992), the folding of procollagen I and transferrin in the mammalian endoplasmic reticulum (Steinmann et al., 1991; Lodish, H. F. & Kong, N., 1991), and translocation of mitochondrial precursor proteins (Rassow et al., 1995). Genetic analysis of immunophilins in lower eukaryotes and bacteria discloses their functions in more detail. Surprisingly, the disruption of multiple immunophilin genes in yeast has shown neither any phenotypic change nor effect on cell growth (McLaughlin, et al., 1992), raising a question of why these proteins are so conserved throughout evolution if cells are not harmed even in the absence of them. It directs speculation to that PPlase activity of immunophilins is required only when cells are exposed to stress conditions like heat shock or starvation. Recent evidence that heat shock induces increases in mRNA levels for immunophilins (Skyes et al., 1993) supports this speculation.

Even though the mechanism of how Mipx contributes to interaction of X-

bacteria in amoebae has not been elucidated despite the fact that Mipx is indeed involved in the initial infection of X-bacteria (Fig. 14), possible interaction mechanisms have been postulated based on the secondary structure, a highly basic pI value and PPlase activity of Mipx.

Cationic lysosomotropic agents such as NH_4Cl and various amines are able to raise the intralysosomal pH and inhibit phagosome-lysosome fusion (Gordon et al., 1980). In addition, cationic polypeptides are known to enhance membrane interactions. Since Mipx is a surface polycation protein with a pI value of 10.02 (Fig. 7), Mipx could be acting like a cationic lysosomotropic agent to inhibit phagosome acidification and phagosome-lysosome fusion. Alternatively, Mipx as a PPlase enzyme could be interacting with vacuolar membrane proteins. For instance, Mipx could interact with H^+ -ATPase on the phagosomal membrane and induce a conformational change of H^+ -ATPase, so that the ability of enzyme to pump H^+ into phagosomes would be inhibited. Another possible role of Mipx in influencing intracellular X-bacteria survival by inhibition of lysosome-phagosome fusion could be through modulating Ca^{2+} transport properties of vacuolar membranes. The reasons that Ca^{2+} could be involved in X-bacteria infection are; i) Ca^{2+} is known to participate in the translocation of lysosomes and endocytic vesicles along microtubules, fusion between vesicles, and signal transduction pathways, ii) calcium transport pathways such as Ca^{2+} channels and Ca^{2+} -ATPase are present on the plasma membrane as well as the membrane of endoplasmic reticulum, iii) FKBP is tightly associated with the Ca^{2+} release channel of the sarcoplasmic reticulum and modulates its gating kinetics (Timmerman et al., 1993; Mayrleitner et al., 1994), and iv) Ca^{2+} induces cellular activation or inactivation of several processes occurring in host cells during parasite infection. The vacuolar membrane containing X-bacteria mixes with

endocytic vesicular membranes presumably derived from endoplasmic reticulum during the infection process. Therefore, it is likely that the vacuolar membrane contains Ca^{2+} -ATPase and Ca^{2+} channels. These proteins, having membrane-buried proline residues that are important for their function, could be the target of the PPIase of Mipx. Therefore, Mipx might block many calcium-related phagocytic cellular activities, including lysosomal fusion with phagosomes and recruitment of cellular organelles by changing the flow possibly occurring around phagosomes.

As mentioned before, Mipx could contact its partner in the vacuolar membrane through the C-terminal region at the very beginning of X-bacteria infection. However, it cannot be ruled out that Mipx may be released from X-bacteria to contact its target or be inserted into the vacuolar membrane to function continuously, since vacuoles containing X-bacteria become bigger by fusion with endocytic vesicles. This hypothesis might be supported by the following experimental evidence: i) In case of *Trypanosoma* infection, PPIase is released from the bacteria and enhances invasion of parasites to mammalian cells (Moro et al., 1995), and ii) Mip of *Legionella* binds and functions as an inhibitor of the activity of protein kinase C in phagocytic cells *in vitro* (Hurley et al., 1993), leading to deregulation of signal pathway proteins in phagocytes.

Surface exposure or release of Mipx from membranes during infection was investigated by immunofluorescence microscopy either with polyclonal antibodies against Mip of *Legionella* or with a mAb against recombinant Mipx of X-bacteria. None of them demonstrated surface exposure or release of Mipx despite the fact that antibodies showed positive results in immuno-blotting. These results could be explained by noting that there are low concentrations of surface-exposed epitopes or limited release of Mipx. Meanwhile, when *E. coli* producing recombinant Mipx were

treated with antibodies against recombinant Mipx under a non-permeable condition, the surface of bacteria was lit up (Fig. 28), suggesting that produced Mipx was localized on the surface in *E. coli*. It could support the result of Fig. 3 that Mipx protein was a membrane protien.

X-Bacteria pretreated with FK506 were able to infect and still maintained multiplication in host amoebae although infectivity was reduced to one third that of untreated X-bacteria (Fig. 14) and *E. coli* transformed with pET-30a/*mipx* were digested inside amoeba (Fig. 29) after experimental infection, suggesting that Mipx participates in early events of the infection processes and other factor(s) may be involved in the interaction between X-bacteria and amoebae. For instance, plasmids of X-bacteria seem to be needed for infection of X-bacteria to amoebae since X-bacteria treated with plasmid-curing agents are digested in phagolysosome (Han & Jeon, 1980). In the case of *Legionella*, molecular genetic approaches have identified a few genes involved in *L. pneumophila* pathogenesis along with the *mip* gene. The *icm* (defects in intracellular multiplication) locus is essential for intracellular multiplication of *L. pneumophila* in human macrophages and is also required for virulence in guinea pigs (Morra et al., 1992; Brand et al., 1994). Another genetic locus, designated *dot* (defects in organelle trafficking), is required for intracellular replication and proper trafficking of cellular organelles during macrophage infection (Berger and Isberg, 1993). The *mak* (defects in macrophage killing) locus is required for macrophage killing (Sadosky et al., 1993). Since several genes appear to be involved in pathogenicity of *L. pneumophila*, the capacity of the *Legionella* to survive and multiply intracellularly is considered to come from a combination of factors.

Since the two genes locus of *Legionella*, *icm* and *dot*, are positioned nearby (Brand et al, 1994), it is worthy of noting 'Pathogenicity islands (Pais)' which is

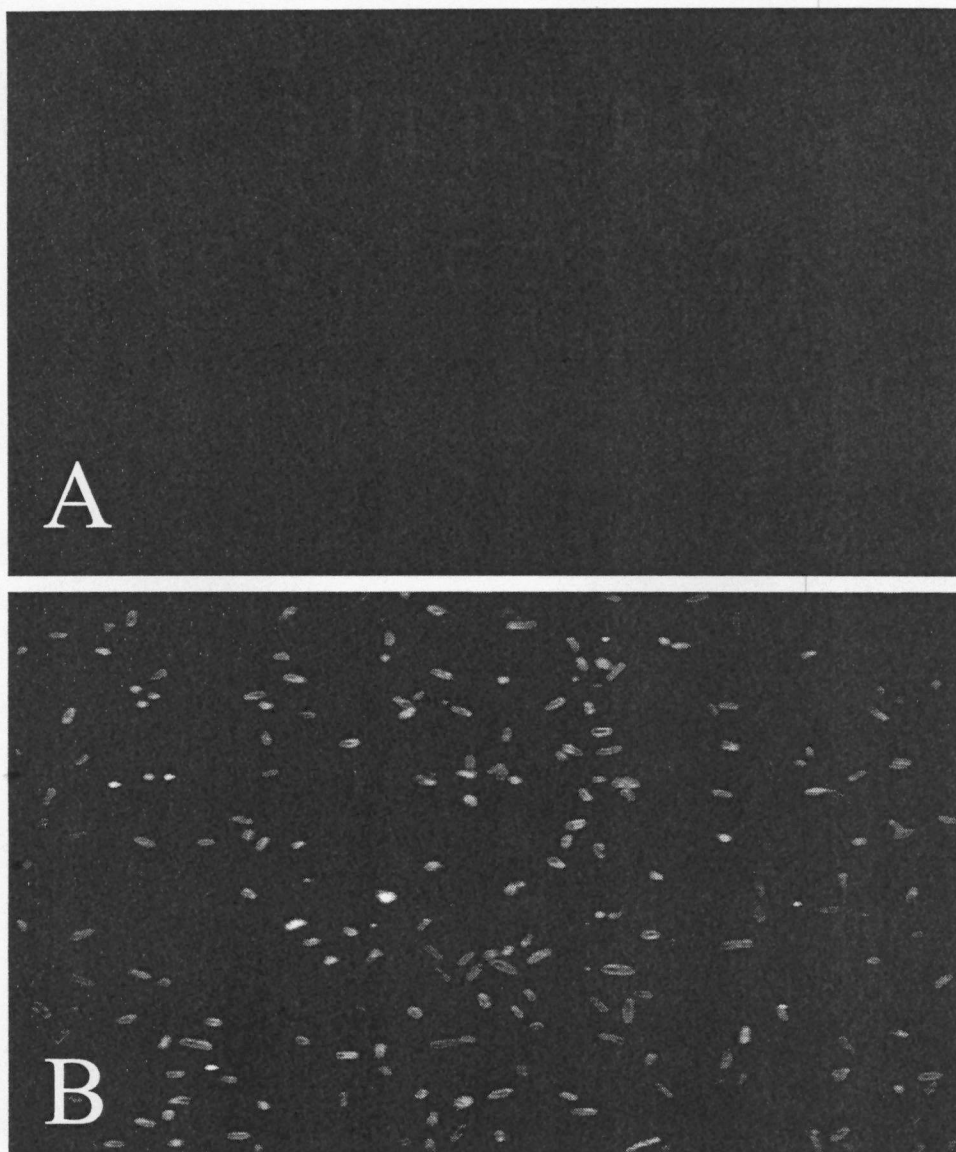


Figure 28. Indirect immunofluorescence micrographs (A, B) of *E. coli* transformed with pET-30a/*mipx*.

Bacterial cells were processed under non-permeable condition with 2% paraformaldehyde solution. As a control in **A**, transformed *E. coli* were stained with pre-immune serum. In **B**, *E. coli* cells were treated with mAb KJX5 against recombinant Mipx.

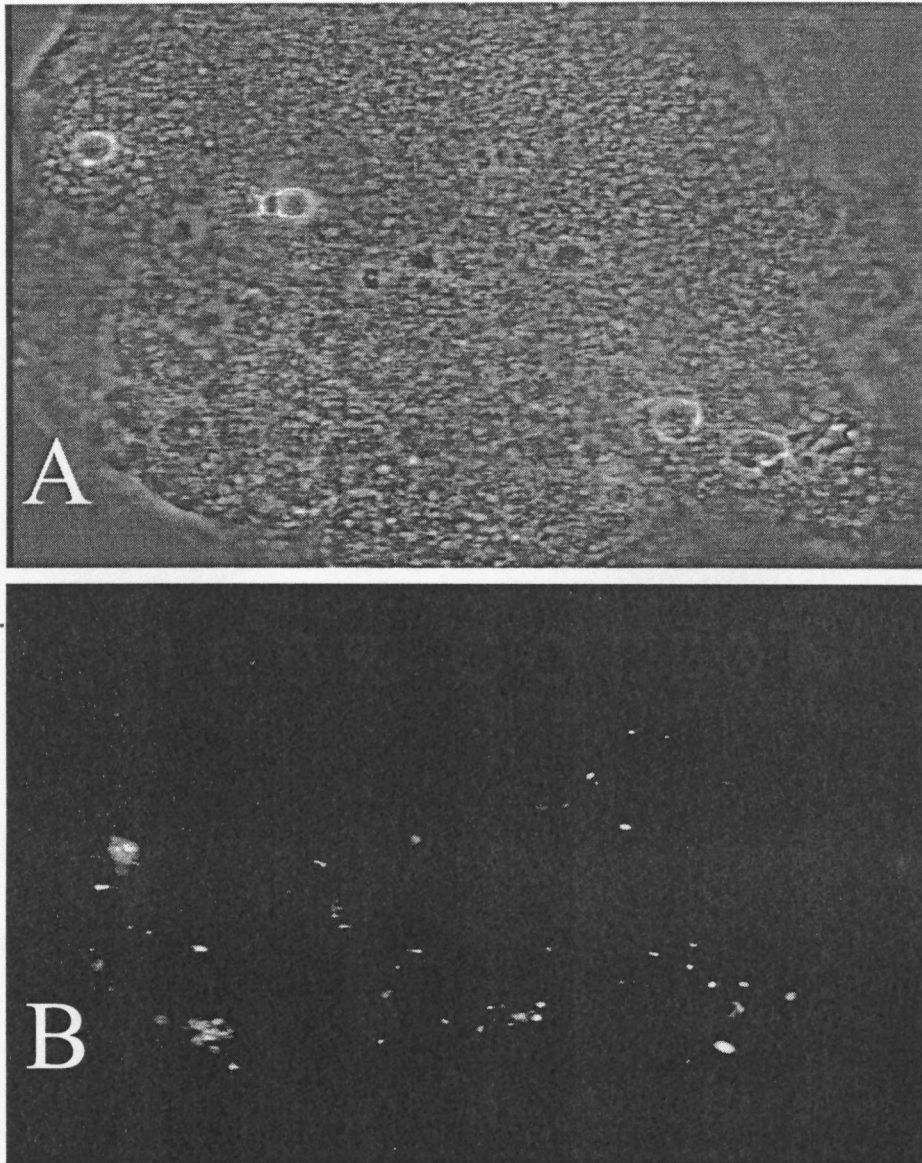


Figure 29. Phase-contrast (A) and immunofluorescence (B) micrographs of amoeba 3 days after experimental infection with recombinant Mipx-producing *E. coli*.

In **B**, amoeba of 3 days after infection with transformed *E. coli* was immunostained with mAb KJX5 against recombinant Mipx. Result showed that most of infected *E. coli* was being digested in amoeba.

recently defined in terms of virulence-associated genes (Hacker et al., 1997). PIs are found in Gram-positive and Gram-negative pathogens, and present in the genome (or plasmid) of pathogenic strains of given species but absent or rarely present in non-pathogenic variants. They occupy particular regions of chromosomes up to 200 kb and carry more than one virulence-associated gene with characters such as the G+C contents different from those of the rest of the bacterial genome and specific DNA sequence flanking them. These islands may represent unique genetic elements which contribute to bacterial virulence and to the genetic flexibility of certain bacterial species.

Among many proteins produced by X-bacteria, the 29-kDa protein (S29x) and 67-kDa heat-shock protein (GroEx) are thought to play important roles in X-bacteria-amoeba interaction (Jeon, 1992; 1995). X-Bacteria synthesize and export a large amount of S29x into the host cytoplasm across bacterial and symbiosomal membranes, but the mechanism for its transport across the two membrane systems is not known. Since S29x is detected in the host's nucleus and the deduced amino-acid sequence of S29x has a stretch of basic amino acids that could act as a nuclear localization signal (Pak and Jeon, 1997), S29x is suspected to be involved in affecting the expression of amoeba's nuclear genes as in the case of the inactivation of S-adenosylmethionine synthetase (SAMS) gene in amoebae by the presence of X-bacteria (Choi et al., 1997). Although the functions of S29x are not known, it seems likely that the production of S29x is induced by symbiosis and that this protein plays an important role in the host-symbiont interaction.

X-Bacteria produce a large amount of a 67-kDa GroEL analog (GroELx) (Choi et al., 1991) and the nucleotide sequence of *groELx* shows a high degree of identity with those of many pathogens including *Legionella* and *Coxiella*. It is assumed that

GroE is involved in host-symbiont interactions and play a protective role for symbionts as molecular chaperones, such as enhancing transport and stabilizing imported proteins (Ohtaka et al., 1992). The level of GroELx in X-bacteria is constantly high, unlike in other symbionts, suggesting a significant role in the survival of X-bacteria inside amoebae. One possible function of GroELx is to help transport of S29x from X-bacteria to amoeba cytoplasm. In addition, GroELx could function as a molecular chaperone in transporting and stabilizing imported proteins from the host amoeba as in other cells.

Even though there is no doubt that the Mip-like protein contributes to the infection of many bacteria including X-bacteria, the question of how the PPLase activity or the cationic nature of Mip-like protein with other molecules functions in the infection of pathogen still remains to be answered. A number of bacterial molecules with enzymatic activity such as phospholipases, proteases, and transferases have been shown to contribute to the pathogenicity of bacteria. Recent studies regarding *Yersinia* infection have shown that a dozen bacterial proteins exhibiting enzymatic activity including tyrosine phosphatase and serine/threonine protein kinase are secreted upon contacting hosts (Cotter and Miller, 1996). Based on accumulated information, certain enzymatic functions of bacterial proteins could play a central role in the survival of intracellular bacteria.

B. Myosin in Amoeba

Myosin is a microfilament (actin)-based molecular motor that constitutes a superfamily of proteins characterized by the presence of a conserved head domain connected by a variety of structurally distinct tail domains. Myosins, traditionally, have been classified into two groups based upon whether they can form thick filaments or not. Myosins-II are the conventional two-headed and filament-forming myosins that play a key role in contraction in muscle cells as well as in basic forms of movement in nonmuscle cells. Unconventional myosins are monomeric and are not capable of forming bipolar filaments.

Myosin-II consists of three gene products, a heavy chain of more than 200 kDa and two light chains of approximately 20 kDa each. A single myosin molecule is a hexameric protein composed of two of each chains. The globular head, called subfragment-1 (S1), of heavy chain contains all of the domains needed for motor functions such as actin-binding and ATP-binding sites. The tail has an α -helical coiled-coil structure, so that it is capable of interacting with the tails of other myosin molecules to form a thick filament. Comparison of the head region in all myosins usually shows a single diagonal line, indicating that the amino acid sequences of head domains are very conserved.

When the amino acid sequence of the head domain in amoeba's myosin is plotted with those in nonmuscle and muscle myosins, very similar graphic-matrix patterns are seen with a single strong diagonal spanning through the entire globular head region (Fig. 24). And, the head region of all myosins is defined by a characteristic GXXGXXG, called P-loop, in the ATP-binding site (Titus, 1993). Lysine and threonine following the last glycine are also conserved in all myosins. In

amoeba's myosin, those amino-acids are well conserved (Fig. 25A). Fig. 26 shows that the tail domain of amoeba's myosin has a rod-like coiled-coil structure, proving that the myosin cloned from amoebae is a true myosin type-II.

Comparisons of amino acid sequence of tail regions in all myosins by graphic-matrix analysis do not show a single line as do the head region. Instead, homologies in the tail region appear in multiple repeating patterns throughout the entire tail (Fig. 24). There are four seven-residue repeating structures in the myosin tail and seven amino acids, the smallest repeating order, form two turns of an α -helix. The first and fourth small hydrophobic amino acids position fall in the interior coiled-coil locations and alternatively charged amino acids of the rest are positioned on the surface of the coiled-coil, which interact with tails of other myosin to form the thick filament. In amoeba's myosin, this 28-residue repeat zones have not been constructed due to the absence of highly conserved proline residue, which is used as a landmark for the end of the head and the beginning of the tail region. The proline residue has been replaced with lysine in amoeba's myosin (residue 840 in Fig. 23). However, as the graphic-matrix analysis between *Amoeba* and *Dictyostelium* shows a multiple repeating pattern (Fig. 24), it is not difficult to predict that the tail domain of amoeba's myosin has the same repeating structure as seen in all myosin type-IIs.

Molecular genetic approaches, such as disruption of the myosin gene by homologous recombination (De Lozanne & Spudich, 1987), expression of truncated myosin gene, and introduction of antisense RNA in *Dictyostelium* (Knecht & Loomis, 1987) have given us valuable information about myosin's involvement in a variety of nonmuscle movements. Myosin is essential for cytokinesis, capping of surface receptors, morphogenetic shape changes associated with developmental processes, intracellular vesicle movements, and modulation of chemotaxis by affecting cell

polarity (Spudich, 1989). The involvement of amoeba's myosin in maintenance of intracellular vesicle and cytokinesis has been shown in Fig. 18 and Fig. 21, respectively.

Phosphorylation has been known to play a central role in the structural and assembly properties of nonmuscle myosin type-II (Tan et al., 1992). Myosin heavy chain phosphorylation occurs on serine and threonine of C-terminal domain of the tail region, and inhibits both filament assembly and actin activation of myosin ATPase. In addition, phosphorylation of myosin heavy chain generates a cell polarity as redistributing myosin molecules. There are phosphorylatable serine and threonine residues at the end of tail in amoeba's myosin (Fig. 23). Further investigation regarding phosphorylation of myosin *in vitro* from cloned amoeba's gene would provide more knowledge about the function of myosin phosphorylation.

Recent years have seen tremendous explosion of information about unconventional myosins. Unconventional myosins have motor domains in the head just like myosin type-II whereas the tail domains are very different among unconventional myosins, which may endow each myosin with a specific function. It is likely that a cell contains multiple isoforms of myosins, each of which plays a unique role in generating actin-based cell movements. It has been known so far that the budding yeast has five myosin genes distributed among three classes, and 26 myosin genes in seven classes have been identified in the mouse (Mermall et al., 1998). Hence, the finding of three isoforms of myosin in amoebae with motor domain-specific sequences (Fig. 27) was expected, considering those of other amoebae of *Dictyostelium* and *Acanthamoeba* (Bement & Mooseker, 1993). With the discovery of new isoforms of unconventional myosins in different cell types, their functions known to be involved in cell movement and locomotion, membrane traffic, organelle movement, sensory, and signal transduction will be extended.

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VITA

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