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Loss of a Strain-Specific Protein by Bacterial Infection in *Amoeba proteus*

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Amoeba proteus에 있어서 박테리아 감염에 의한 변이주 특이성 단백질의 손실

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요 약

Amoeba proteus xD strain에서 추출한 공생박테리아를 tD strain에 감염시키고, 숙주의 박테리아 감염으로 인한 변이주 특이성 단백질의 손실을 2차원 전기영동에 의해 탐지하였다. 유도 식작용에 의한 실험감염 50일 만에 숙주인 아메바는 tD strain 특이성 단백질을 손실하였으며, 이는 27°C 배양에 의해 감염 박테리아 및 xD strain 특이성 단백질을 제거한 후에도 재합성되지 못하였다. 이 시기면 숙주인 아메바는 박테리아에 완전히 의존한 것으로 판명되었다.

이상의 결과 및 Lorch와 Jeon (1981, Science 221:549)의 결과를 볼 때 감염된 숙주핵이 감염되지 않은 아메바 원형질과 양립하지 못하는 것이나 숙주의 박테리아에 대한 의존 유발은 박테리아 감염에 의해 세포 특이성 유전인자의 비가역적인 불활성화 또는 손실로 인한 것이 분명하다.

INTRODUCTION

We previously reported detection of cell-specific proteins present in tD and xD strain (Jeon, 1973) of *Amoeba proteus* (Ahn, 1983; Ahn and Jeon, 1983). By two dimensional gel electrophoresis, one prominant polypeptide with an isoelectric point (pI) of 5.5 and a molecular weight of 29,000 was shown in the cytoplasm of symbiont-containing xD-strain, but not in tD-strain. Nonsymbiotic tD strain contained a cell-specific protein whose mole-

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cular weight and pI was 45,000 and 5.9, respectively.

xD strain was originally derived from tD strain in 1966 by spontaneous infection with an unidentified rod-shaped bacteria (Jeon and Lorch, 1967). The infected amoebae not only overcame the initially observed harmfull effect of the infective bacteria, but also the nuclei of the host became dependent on their symbionts for survival (Jeon, 1972; Jeon and Jeon, 1976; Lorch and Jeon, 1980). Through the organismic association over the 15 years symbiotic bacteria had also been changed. The harmful effects of the bacterial infection such as in clonability and sensitivity to starvation that were shown in the initial infection are not detectable any more (Ahn and Jeon, 1979). When the bacteria of xD strain were purified and newly introduced into tD strain, the host amoebae showed temperature sensitivity in 200 generations (Jeon and Ahn, 1978). A study of nuclear transplantation showed that nuclei of the newly infected amoebae were incompatible with the cytoplasm of uninfected tD strain in 10 to 15 cell generations (Lorch and Jeon, 1981). The nucleus of the host underwent a kind of irreversible change.

In this paper we are reporting the critical time for the irreversible loss of a cell specific protein that was determined by two dimensional gel electrophoresis of the newly infected tD amoebae. The significance of this finding and the possible mechanisms for the loss of ability to synthesize cell specific protein were discussed.

MATERIALS AND METHODS

1. Cell Culture

Mass culture; Amoebae were grown in a modified Chalkley's medium with axenically cultured *Tetrahymena pyriformis* as food organisms (Ahn, 1983). *Tetrahymena* was cultured in a medium containing 0.2% liver concentrate and 2% proteose peptone. The amoebae were fed three times a week with fresh medium. Cells were cultured at 23°C to a density of 2×10^5 cells/dish (18cm diameter, 2cm in depth). To test temperature sensitivity or to eleminate infected bacteria from the experimentally infected amoebae, cells were grown at 27°C (Jeon and Ahn, 1978).

Single cell culture; By using fine tipped mouth pipette, healthy amoebae were picked up individually from the experimental group and placed singly in Syracuse watch glass containing 1.0 ml medium. Healthy amoeba attached well to the bottom of the dish and was conspicuous in cytoplasmic streaming. For each experiment, 24 amoebae from each group of experimentally infected amoebae were cultured. Single cell culture was also fed three times a week. On the day of feeding, amoebae in each dish were counted. The progeny of any amoeba that continued to divide for up to 3 weeks was considered to be forming a viable clone (Jeon and Ahn, 1978).

2. Experimental infection

Isolation of symbiotic bacteria; Healthy xD amoebae were harvested from mass culture,

and their symbiotic bacteria were isolated at 4°C as follows. Washed amoebae were centrifuged for 25 seconds at 170g. To a volume of packed amoebae (3.8×10^5 cells/ml), equal volume of ice-cooled 0.02M tris-HCI buffer pH 7.4 was added. The cell suspension was homogenized with mild pressure in a glass homogenizer. The lysate was centrifuged for 5 min at 350 g and supernatant was centrifuged again for 10 min at 10,000 g. The supernatant fraction of the second centrifugation was used as protein sample for gel electrophoresis and the pellet which was containing bacteria and a little cell debris was frozen and thawed twice to destroy cell membranes and organelles. After removing cell debris by centrifugation for 5 min at 10,000g, bacteria were further purified by centrifugation in sucrose step gradient. Bacteria were collected from 30% and 30/40% boundary and was washed three times with Chalkley's solution.

Infection by induced phagocytosis; For experimental infection, isolated symbiotic bacteria were introduced into tD amoeba by induced phagocytosis. Purified bacteria were preincubated for 20 min in Chalkley's solution containing 0.05% poly-L-lysine to make random aggregate. The random aggregates of bacteria were collected by centrifugation for 5 min at 2,000 g. The aggregated pellet was washed and suspened in Chalkley's solution to a density of 6×10^8 bacteria/ml. This suspension was added into the culture dish containing well attached amoebae. Amoebae were induced to phagocytose aggregated bacteria for 2hr at room temperature. Then the amoebae were washed repeatedly with fresh medium to remove remaining bacteria, and fed with *Tetrahymena* from a day after infection.

3. Two-dimensional gel electrophoresis

Electrophoresis was performed according to the method of O'Farrel (1975) with minor modifications. Isoelectric focusing (IEF) gel was prepared using pH 3-10 and pH 6-8 ampholines (LKB instruments, Inc.) as carriers and polymerized in glass tubing (130 × 3 mm inner diameter). One volume of protein sample was mixed with another volume of lysis buffer containing 9.5M urea, 2% ampholine (comprised of 1.65% pH 6-8 and 0.4% pH 3.5-10 ampholines), and 5% mercaptoethanol. In each gel, 400μg protein was applied. IEF was carried out for 20hr at 400V followed by 1hr at 800V. SDS polyacrylamide gel electrophoresis in the second dimension was carried out in 1.5mm-thick slabs of 8~14% gradient gels containing 0.1% SDS. The IEF gels were placed over slab gels after equilibration for 1.5hr in the treatment buffer at room temperature. The IEF gels were sealed in place with 1% agarose in treatment buffer, and electrophoresed for 6hr at 20mA per slab. Molecular weight standards were loaded in a slot made at the edge of the sealing gel. After the completion of electrophoresis, gels were fixed and stained for 2-3hr in a solution containing 0.125% Coomassie brilliant blue, 50% methanol and 10% acetic acid. Gels were destained for 1hr in 50% methanol and 10% acetic acid, then in 10% methanol and 5% acetic acid until the background disappeared. To enhance staining sensitivity, silver staining method of Merril *et al* (1981) was also applied.

RESULTS

1. Cell culture of newly infected amoebae

The clonability of the newly infected amoebae at 27°C was compared with those of nonsymbiotic and symbiotic amoebae (Table 1). At this temperature the nonsymbiotic tD amoebae continued to multiply indefinitely, but symbiotic xD strain ceased to divide in 10 days, and died out within 14 days (Fig. 1).

The amoebae that had been infected for shorter period of time divided and survived

Table 1. Growth and clonability of amoebae at 27°C that have been infected for varing period of time

Amoebae	Time after infection	Cells failing to form clones	
		%	*No
tD strain	Nonsymbiotic	0	0/38
Experimentally infected	50 days	22.2	4/18
	4 months	27.0	13/48
	12 months	60.0	27/45
xD strain	over 18 years	100.0	41/41

* The numbers represent cells cultured in two seperate experiments at different times.

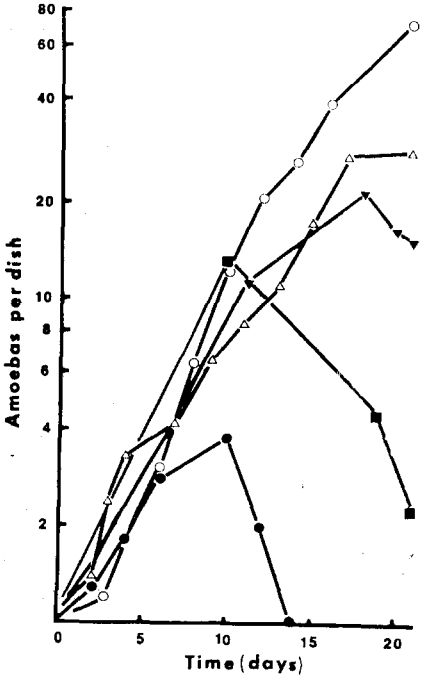

Fig. 1. Growth curves of amoebae at 27°C. tD amoebae, open circles; xD amoebae, solid circles; and tD amoebae infected by bacteria for 50 days, open triangles; for 4 months, solid triangles; and for 1 year, solid squares. Each point represents the average of 18 to 48 singly grown cells.

longer than the amoebae that had been infected for longer period of time (Fig. 1). When the infected amoebae were grown at 27°C for long period of time, they all died out. In single cell culture the amoebae that had been infected for 12 months have lost clonability to 40%.

After growing the newly infected amoebae at 27°C for 21 days till none of the infected bacteria detectable under the phase contrast microscope, the survived amoebae were transfered individually to Syracuse dishes and grown singly at room temperature for 2 weeks. Then the amoebae from each single cell culture were ruptured on the glass slide and studied under the microscope. All of the amoebae that divided to form clones contained symbiotic

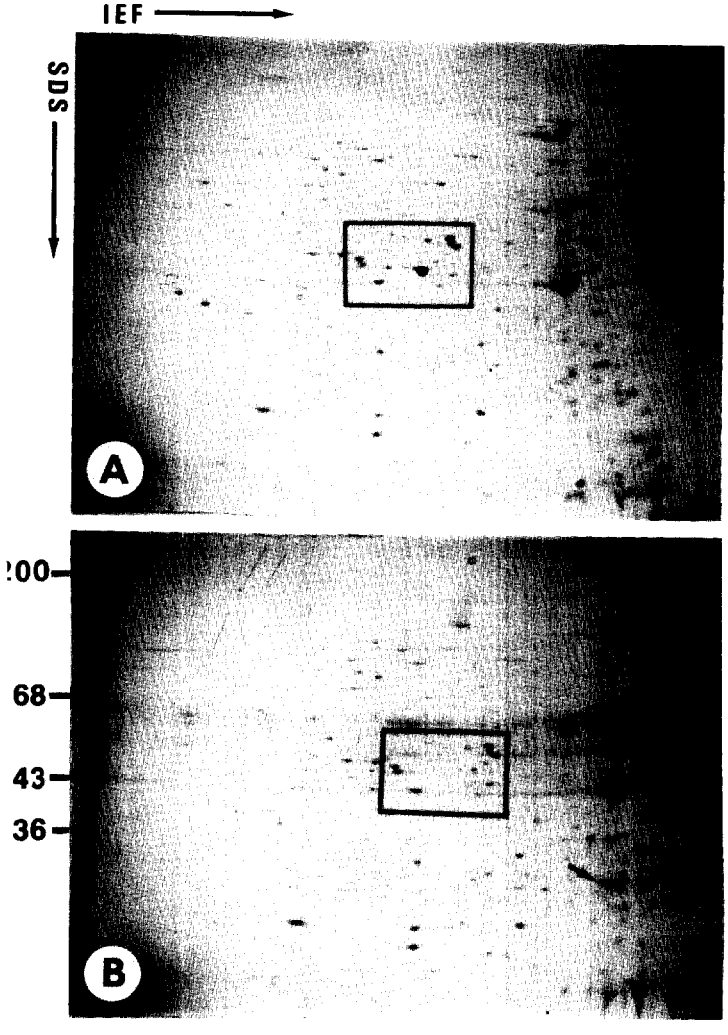

Fig. 2. Two-dimensional electrophoretic patterns of cytosol proteins of tD (A) and xD (B) strain, after staining with Coomassie brilliant blue. tD strain-specific protein can be identified within the squared area, and xD strain-specific protein is indicated by arrow.

bacteria in the cytoplasm. None of the infected amoebae that survived could be free of infected bacteria. From this result it was clear that the infected hosts even at 50 days of infection, definitely obtained dependence on infected bacteria for their survival.

2. Irreversible loss of tD specific protein by bacterial infection.

Presence of the two strain-specific proteins of tD and xD amoebae were reconfirmed by two dimensional gel electrophoresis (Fig. 2). Stained density of tD strain-specific protein was more prominant when the amoebae were grown at 27°C. Focusing the proteins with Ampholine of pH 6~8 resolved better for the tD specific protein than pH 5~8 since the narrower range of the pH carrier broadened the distance of the near-by proteins.

In order to determine the critical time for the loss of tD specific protein in the newly infected amoebae, protein samples of the amoebae that had been infected for 5, 15, 30, and 50 days were electrophoresed (Fig. 3). The decrease in tD cell-specific protein was prominant along with the increase in length of infected age. In Fig. 3d, tD cell-specific protein was not detectable in the amoebae that had been infected for 50 days. This cell-specific protein was not detectable in none of the amoebae that had been infected for longer period than 50 days (Data are not shown). In the newly infected amoebae, xD cell-specific protein was detectable from the 6th day of infection and the spot was clearly visible at the 9th

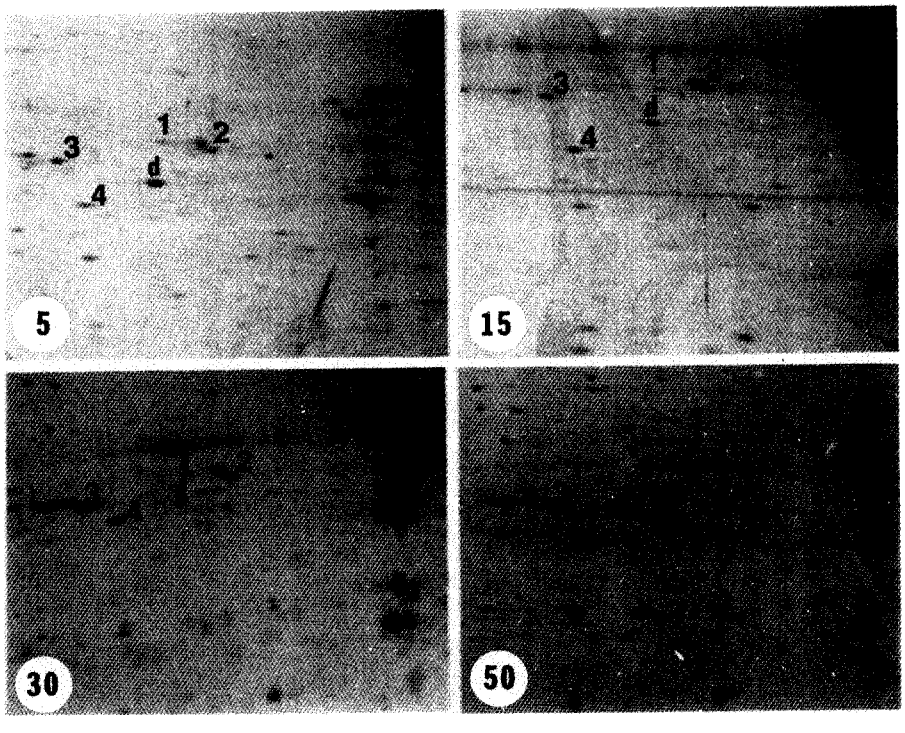

Fig. 3. Selected areas of two-dimensional electrophoretic gels of proteins from the amoebae newly infected for 5, 15, 30 and 50 days. In order to identify tD cell-specific protein (d) in the figure, reference proteins are numbered 1 to 4.

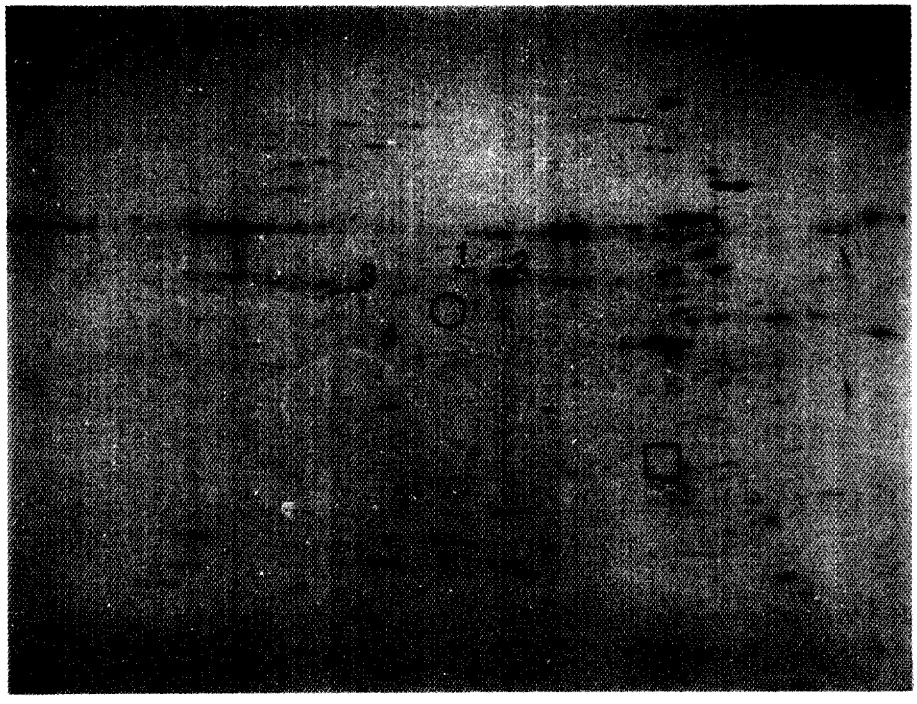

Fig. 4. A protein pattern of the newly infected amoebae cultured at 27°C for 14 days. xD cell-specific protein was completely eleminated (squared area). Note the tD cell-specific protein is not detectable (circled area).

day of infection.

Since the loss of tD cell-specific protein from the host was due to the presence of infected bacteria and the xD cell-specific protein in the cytoplasm, both of these inhibitory factors were removed by growing the infected amoebae at 27°C, and the possibility of the reversible synthesis of the protein was tested. The newly infected amoebae that had lost tD cell-specific protein were grown at 27°C long enough after eleminating the infected bacteria untill none of the xD cell-specific protein was detectable. In the gel shown in figure 4, tD cell-specific protein was not detectable. From this experiment it was clear that the loss of the ability to synthesize tD cell-specific protein was irreversible.

DISCUSSION

The symbiotic bacteria appears to change by prolonged endocellular symbiosis. As noted early many of the ill effects of bacteria are completely undetectable (Ahn and Jeon, 1978). Comparing with the clonability of the newly infected amoebae shown in 1978, duration

for obtaining temperature sensitivity has been shortened to half (cf. Table I with Table I of Ahn and Jeon, 1978).

In considering the time table for the development of symbiosis in this amoebae (review; Jeon, 1983) determination of the time period for the loss of tD strain-specific protein has enough significance to understand the molecular mechanism of organismic association. In the results presented above, dependence of the host on the infected bacteria and the loss of a host-specific protein occured within 50 days of bacterial infection. This time period corresponds about 24~25 cell generations of amoebae. The nuclei of the newly infected amoebae at the age of 10~15 cell generations were found incompatible with the cytoplasm of uninfected tD cell (Lorch and Jeon, 1981). Compared to the homotransplanted nuclei that were completely compatible with tD cytoplasm showing 96.3% clonability, the nucleus of the infected host underwent rapid change. Our results convince that the incompatibility of the nuclei of the infected amoebae is due to the irreversible loss of the synthetic ability of tD cell-specific protein. In xD amoebae, bacteria or the xD specific protein which are in the cytoplasm may compensate the function of the lost tD cell-specific protein. Inability to synthesize the cell-specific protein in the newly infected amoebae may be further confirmed by utilizing labeled amino acids.

Yet, we do not know how the amoebae lost the cell-specific protein by bacterial infection. Since the symbiotic bacteria carry two kinds of plasmids (Han and Jeon, 1980), it may be due to the irreversible inactivation of the cell-specific gene or the loss of the gene from the host chromosome. Possibility of gene transfer by the plasmids of infective parasitic and symbiotic bacteria was postulated in various organisms (Zambryski *et al.*, 1983), where the gene reshuffling such as insertion, interruption of structural genes, activation of gene expression or stimulation of gene deletion, was mediated by transposable elements or insertion sequences of the plasmid. *Agrobacterium* that induce crown gall by infecting dicotyledonous plants is known to carrry plasmid containing transposon (Ream and Gordon, 1982; Zambryski et al., 1980). Transfer of genetic information carried by this genetic element was well documented. Now they consider the transposon as a natural genetic engineer as it transfer DNA to the plant (Zambryski *et al.*, 1983). There are a few circumstancial evidences in favor of gene transfer in the fish host and ***Photobacter leiognathi*** (Martin and Fridovich, 1981), and in insect host and its bacteria-like endosymbionts (Schwemmler, 1983).

For the symbiotic bacteria, inducing the loss of a gene may be a specific way of adaptation. Further study of this amoebae will illustrate the possible molecular mechanism not only for the development of endocellular symbiosis but also for the origin of cell organelles.

SUMMARY

By two-dimensional gel electrophoresis loss of a cell-specific protein was detected in tD

strain of *Amoeba proteus* that had been infected by symbiotic bacteria extracted from xD strain. In 50 days of experimental infection by induced phagocytosis the host amoeba lost the ability to synthesize the tD cell-specific protein even after removal of the infective bacteria and xD cell-specific protein by growing the amoebae at 27°C. By this time the host amoebae were obligately dependent on the bacteria. From these and other results (Lorch and Jeon, Science 221 : 549), it is clear that the incompatibility of the infected nuclei with the cytoplasm of the uninfected amoeba and the obligate dependence of the host on bacteria are due to the irreversible inactivation or the loss of the cell-specific gene by bacterial infection in this amoeba.

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