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Susceptibility of *Bacillus subtilis* SNU816 to bacteriophage SP816 during growth and sporulation.

Oh-hyoung Lee and Zoo-shik Lee
(Departmment of Microbiology, College of Natural Science, Seoul National University)

성장 및 포자형성 중인 *Bacillus subtilis* SNU816의 SP816 박테리오파아지에 대한 감수성에 관하여

李 吾 炯 · 李 周 植
(서울대학교 자연과학대학 미생물학과)

ABSTRACT

The changes of susceptibility of *Bacillus subtilis* SNU816 to bacteriophage SP816 were investigated. When *B. sutilis* SNU816 cells were infected by the phage during vegetative growth, rapid lysis was observed. But when they were infected after late logarismic phase, they were resistant to phage infection. Since asporogenic culture of this strain was invariably lysed regardless of time of infection, the arrest of phage multiplication seemed to be caused by sporulation. In reality, the arrest of phage multiplication occurred at early stage of sporulation. Electron microscopy revealed that the arrest of phage multiplication occurred just prior to or during septum formation (stage II of sporulation).

INTRODUCTION

Bacillus subtilis SNU816 was firstly described by Lee (1974). But little was studied on the bacteriophages related to this strain. Only one report verifying the existance of a bacteriophage infectious to this strain had been published (Lee, 1978). In this communication, we report the experimental results concerning the infectivity of bacteriophage SP816 during growth and sporulation of *B. subtilis* SNU816.

B. subtilis is spore-foming, thus is one of the most attractive bacteria to the biologists investigating the structural, physiological, and genetical mechanisms of cell differentiation because of its simplicity compared to any other higher organism.

But, in reality, even the bacterial sporulation is a quite complex process undergoing sequential changes under highly ordered genetic control. Although much attention has been concentrated to the sporulation process, the detailed mechanism by which vegetative cell is converted into dormant spore is so far not well understood.

Bacteriophages infectious to this spore-forming microorganism are remarkablely useful in studies on the mechanism of initation and subsequent control of sporulation process (Szybalski *et al.*, 1969), for they provide good examples of gene selectivity and regulation which govern sequential gene expression at the transcriptional level. Initiation of spore development implies, in the first place, a switch from the expression of vegetative genes to those involved in sporulation. This gene selectivity has been reported to be mediated by change in RNA polymerase (Losick and Sonenshein, 1969; Greenleaf *et al.*, 1973). This conclusion is primarily based on the observations that though RNA polymerase

purified from vegetative cells of *B. subtilis* was able to transcribe phage ϕe DNA, RNA polymerase from the sporulating cells could not do this.

Therefore, a phage whose replication is blocked at early stage of sporulation can provide excellent tools in studying molecular biological mechanism of gene expression that occurs during sporulation. In order to find out such phage, we investigated the susceptibility of *B. subtilis* SNU816 to phage SP816 during its growth and sporulation.

MATERIALS AND METHODS

Microorganisms

Bacterial host: *B. subtilis* SNU816 (Lee, 1974) stock-cultured in nutrient agar slant was used as host throughout the experiment.

Phage: bacteriophage SP816 isolated from rice-straw (Lee, 1978) was used in this experiment.

Preparation of phage stock solution:

A fresh nutrient agar slant with condensed water was inoculated with one-loopfuls of both host and phage, and cultured at 37°C overnight. The clear condensed water in agar slant was used as phage inoculum.

High-titer phage stock solution was prepared as follows. An overnight culture of *B. subtilis* SNU816 in nutrient broth was diluted 1:20 into fresh nutrient broth (9.5ml volume) and was shaken cultured at 37°C until it reached mid-log phase (A_{600}=0.5). To this cultlure 0.1ml of phage inoculum was added and allowed to shake at 37°C until lysis was completed. 1ml of this lysate was used as phage stock solution at next cycle of lysis. As the numbers of lysis cycle increased, gradually higher-titer phage stock solutions were obtained. All of these phage stock solutons were purified by centrifugation, and used as phage incoculum to be 5% volume of culture medium.

The titer of each phage stock solution was determined by plating 0.1ml of each one-tenth dilutions on agar plate preinoculated with 0.1ml of bacterial spore suspension (about 10^8 spores/ml).

After overnight incubation, the number of plaques formed was counted.

Media

Nutrient sporulation medium phosphate (NSMP) was used for sporogenic culture. Its compositions were as follows (Fortnagel and Freese, 1968): Nutrient broth 8g/l, $FeCl_3$ 0.01mM, $CaCl_2$ 0.7mM, $MnCl_2$ 0.05mM, $MgCl_2$ 1.0mM, potassium phosphate buffer 0.1M, pH 6.5. $FeCl_3$, $CaCl_2$, $MnCl_2$, and $MgCl_2$ were dissolved seperately in distilled water and autoclaved. These were added to sterile buffered nutrient broth.

Asporogenic culture medium was simply prepared as follows. Complete NSMP medium was autoclaved at 121°C for 15 min and centrifuged to remove resulting ppt of salts. The supernatant was sterilized by membrane filtration. This filtrate was used as asporogenic culture medium. Sporulation was severely suppressed (less than 10%) in this medium because of deficiency of salts.

Cultivation

All cultivations were carried out at 37°C with shaking at a speed of 120rpm in air-controlled shaker.

Partially synchronized culture was achieved by three transfers of cells which had reached mid-log phase (A_{600}=0.5) on NSMP medium to the same fresh medium. To investigate the susceptibility of host to phage, cells were cultured primarily in Erlen-meyer flasks. And when the culture was reached to appropriate turbity, 9.5ml fractions of this culture solution were made into L-shape sterile test tubes simultaneously, and cuultivation was continued. The size of test tubes used had been carefully selected suiting to spectrophotometer and thus allowing direct measurent of turbidity to be possible. During cultivation, each test tube was

taken out of incubater for a time being, and after measurement of turbidity, the culture tube was reincubated. By using this method, rapid measurement of turbidity was possible (one sample could be measured within 10 sec).

To investigate phage-induced lysis, 0.5ml of phage stock solution with appropriate phage titer was inoculated into one of culture tubes at appropriate time of cultivation, and the turbidity was compared with that of uninfected control.

To investigate sporulation-related events, one culture tube was taken out of incubator, and after absorbance was checked, one-loopfuls of two specimens were made on slide glasses for either spore stain or counting refractile cells. The remaining culture solution was transferred to preweighed cap-tube and centrifuged (1500× g, 10min). The supernatant and the pellet were subjected to determination of extracellula protease(ECP) activity and dipicolinic acid (DPA) content, respectively.

Measurement of bacterial growth

Spectronic 20 colorimeter (Bausch & Lomb) was used to measure turbidity at 600nm

Measurement of sporulation

Percent sporulation was measured either by counting the refractile cells under phase contrast microscopy (Nikon apophot multipurpose microscope) or by spore stain (Bartholomew and Mittwer, 1950).

Measurement of dipicolinic acid (DPA)

DPA content of cell was determined by methods described by Janssen *et al.* (1958) as follows. About 10ml of culture solution was transferred to preweighed cap-tube and centrifuged (Hitachi 20PR-5, Automatic High Speed Refrigerated Centrifuge) at 1500×g for 10min. The dry weight of pellet was determined by subtracting the weight of captube from total (dried pellet+cap-tube) weight. After weighing, 10ml of D.W. was added, and autoclaved for 15 min, at 15lb/in^2. The suspension was cooled, acidified with 0.2ml of 1.0N acetic acid, and left at room temperature for lhr. After centrifugation at 1500×g for 10min, 4ml of supernatant was mixed with 1ml of color reagent consisting of 1% $Fe(NH_4)_2(SO_4)_2 6H_2O$ and 1% of ascorbic acid in 0.5M acetate buffer (pH5.5). Absorbance was measured at 440nm (Cecil CE272 Linear Readout Ultraviolet Spectrophotometer) against blank (4ml of supernatant plus 1ml of DW) within 2 hrs after the addition of color reagent.

Measurement of extracellular protease (ECP) activity

ECP activity during growth and sporulation was measured as described by Ogrydziak and Scharf (1982) with slight modification as follows. 2.5ml of 0.6%(W/V) casein in 10mM phosphate buffer (pH6.8) was reacted with 0.5ml of cell-free culture solution at 37°C for 10min in waterbath. The reaction was stopped by adding 2.5ml of protein precipitating reagent (0.11M TCA+0.22M sodium acetate+0.33M acetate). After further incuvation at 37°C for 20min, reacting solution was centrifuged at 1500×g for 10min. And the optical density of supernatant was measured at 275nm against air or water with spectrophorometer (Gilford Spectrophotometer 250). Blank was prepared with same volume of reaction sulutions by converting the reaction sequences as follows. Substrate was treated with protein precipitating reagent and allowed to stand at 37°C for 10min, and followed by addition of enzyme solution. After further incuation for 10min, reacting solution was centrifuged and measured.

Transmission Electron Microscopy (TEM)

To find out the exact stage of sporulation and phage-host relationship, phage-infected cells were observed under TEM. The specimens for TEM were prepared as follows: Phage infected culture was centrifuged (1500×g, 10min) after 50min of phage infection. The pellet was washed once with 0.01M phosphate buffer (pH7.0) and prefixed with 3% glutaraldehyde. Details of follo-

wing treatments and methods were as same as those described by Lim *et al.* (1983).

Chemicals

DPA, ascorbic acid, and acetic acid(glacial) were purchased from Sigma Chemicals Co., and casein (Hammarsten) was purchased from ICN Pharmaceuticals. And all other salts (S.P.C. GR grade) were purchased from Shinyo Pure Chemicals Co.

RESULTS AND DISCUSSION

Multiplication of SP816 in unsynchronized culture of *B. subtilis* SNU816

One loopful of slant culture of *B. subtisis* SNU816 was inoculated into 10ml of sterile nutrient broth and cultured overnight with shaking. This culture broth was transferred into 200ml of fresh nutrient broth and cultured again. When the culture reached to A600=0.1, 9.5ml of fractions were made into L-shaped sterile test tubes aseptically, and cultivation was continued. At appropriate time interval, each culture tube was infected sequentially with 0.5ml of low-titer phage stock solution (about 10^7 PFU/ml)

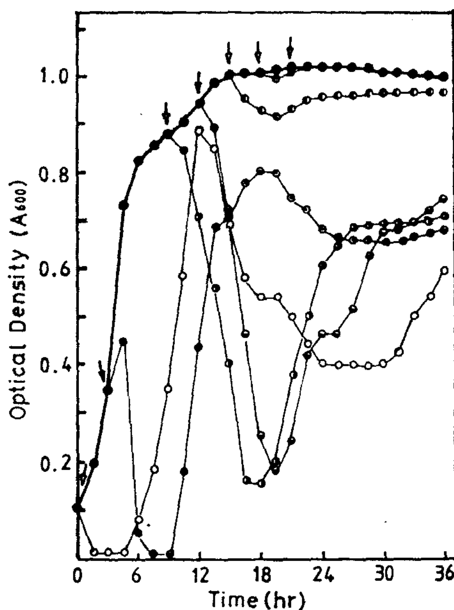

Fig. 1. Time courses of cell growth and phage-induced lysis. Unsynchronized culture of *B. subtilis* SNU816 was infected with low-titer phage stock solution at the time indicated by arrows
●——●; growth of uninfected cells
All others; growths of infected cells

The profiles of growth and lysis were indicated at Fig. 1. When cells were infected at early growth phase, they were rapidly and almost completely lysed. But when they were infected at later phase of growth, the rate and extent of lysis were gradually decreased and almost no lysis was observed after 18hrs of cultivation. These results indicated that multiplication of phage was highly dependant on the state of host. But it was so far not clear whether arrest of phage multiplication was caused by sporulation or by inactive metabolism of host.

Meanwhile, an unusual phenomenon was observed. As Fig. 1 denoted, the populations of cells of phage-infected tubes fluctuated in a similar pattern. Once lysis was ceased, second phase of growth was resumed, and followed by second phase of lysis. These fluctuations of dual growth and lysis seemed to be quitely regulated since the height of each wave was gradually decreased according to the culture time. The reason for this unusual phenomenon is so far not clear. And an intensive research to find out the real cause of this unusual phenomenon is now being performed in this laboratory.

Multiplication of SP816 in asporogenic culture of *B. subtilis* SNU816

To determine whether the failure of phage multiplication in cells of stationary phase (Fig. 1) was caused by sporulation of host or not, cells were cultured in asporogenic culture medium and infected with high-titer phage stock solution (7.2×10^9 FPU/ml). Sporulation in this medium was markedly reduced (data not shown). The host was cultured several times in this medium before phage infection to be freed of salts carried by host itself, and finally transferred to L-shaped test tubes.

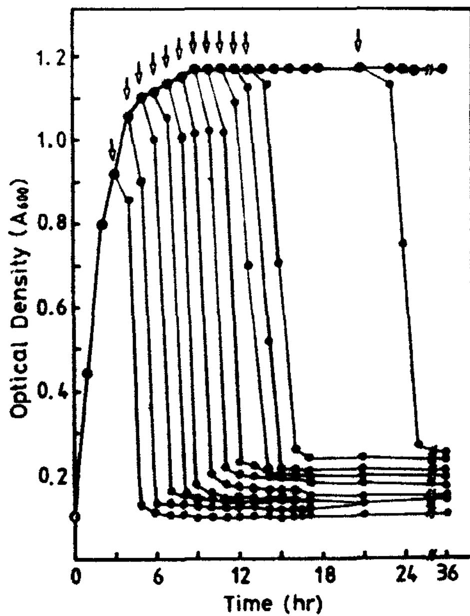

Fig. 2. The course of phage SP816 infection in asporogenic culture of *B. subtilis* SNU816. Arrows indicate the time of phage infection.

As was represented at Fig. 2, cells in this medium were lysed regardless of time of phage infection. Even the cells infected after 21hrs of cultivation were lysed rapidly.

The tribial amount of cells remained unlysed were proved to be spores when they were examined by spore stain at 36 hrs of cultivation (data not shown). Therefore it was somewhat clear that insusceptibility of cells to the phage infection might be acquired by sporulation.

Multiplication of SP816 in partially synchronized culture of *B. subtilis* SNU816

Since it was revealed that the blocking of phage multiplication was caused by sporulation, an attempt to find out the exact stage of sporulation at which phage multiplication might be prevented was made. To support as optimum sporulation as possible, cells were cultured in NSMP, and to achieve coordinate sporulation, cells were partially synchronized as described in materials and methods.

9.5ml fractions of partially synchronized culture solution from Erlen-meyer flask were made into L-shaped test tubes at $A_{600}=0.5$, and 0.5ml of high-titer phage stock solution was inoculated into each culture tube at 1hr intervals. To find out the exact stage of sporulation, stage specific events such as ECP production, DPA accumulation, appearance of refractivity, and liberation free spores were followed during cultivation, and the cells at each stage were observed by electron microscope after 50mins of phage infection.

As was shown in Fig. 3, partial synchronization was evident, for all the sporulation specific events investigated were completed within 3hrs. Remarkable change in susceptibility of host took place within one hour. Namely, the cells infected at 3hr of cultivation were lysed completely, but the cells infected at 4 hr of cultivation were only slightly lysed and majority of

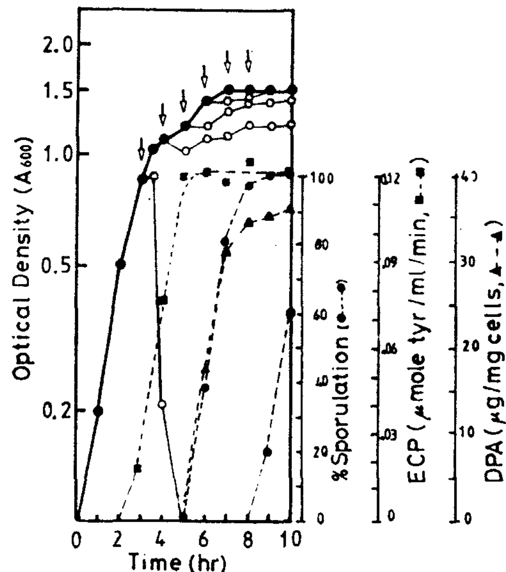

Fig. 3. Time courses of phage SP816 multiplication and sporulation specific events during cultivation of *B. subtilis* SNU816. Partially synchronized culture of host was infected with high-titer phage stock solution at the time indicated by arrows.

●——●; growth of uninfected cells
○——○; growth of infected cells
■……■; extracellular protease activity
●……●; refractivity
▲……▲; DPA content
⊙……⊙; free spores

cells were resistant to phage infection. Still less cells were lysed thereafter. The appearance of phage-insusceptible cells was well coincided with that of extracelluar protease (Fig. 3, ■…■). It was about 2.5 hrs before the appearance of refractivity and DPA (stage V of sporulation), and about 6 hrs befroe liberation of free spores (stage VII of sporulation). Sporulation in this case was completed somewhat faster, for usual liberation of free spore occurs 8-10 hrs after initiation of sporulation (Hanson *et al*, 1970). Since maximum synthesis of ECP normally occurs just before or during sporulation (Priest, 1977; Hanson *et al*, 1970), it seemed quite true that the blocking of phage multiplication occurred early in sporulation, as it were, just before or during stage II of sporulation where septum formation occurs. In fact, E.M. revealed that cells infected at 3 hr of cultivation were predominantly vegetative forms and most of them were undergoing lysis (Plate 1a). In contrast, cells infected at 4 hr of cultivation had a membraneous structure in their cytoplasm showing inward folding of the cell membrane to form fore spore septum (data not shown), or had a completed septum (Plate 1b). Cells in this stage were resistant to phage infection as the sound cytoplasm indicated. All the cells behind this stage were also resistant to phage infection (Plate 1c∼g).

All of these results indicated that the arrest of multiplication of phage SP816 took place in stage I or II, just prior to or during septum formation. A loss of the ability of cells to produce lytic phage during sporulation has been reported in studies with *B. subtilis* (Sonenshein and Roscoe, 1969; Kawamura and Ito, 1974). Sonenshein and Roscoe (1969) observed that asporogenic mutants of *B. subtilis* 3610 continued phage production when infected with phage ϕe in the post-logarithmic growth phase while wild-type cells infected after To produced fewer phage particles than growing cells, and infection at T_5 to T_6 resulted in incorporation of phage genome into the spore. Kawamura and Ito (1974), on the other hand, reported that phage ϕe could multiply in *B. subtilis* 168 undergoing even later stages of sporulation and the synthesis of ϕe-specific mRNA was observed in cells infected more than an hour after the time of inhibition of DNA synthesis. Our results were quite similar to those of Sonenshein and Roscoe (1969). Furthermore, as Plate 1f denoted normal adsorption occurred even in the cells bearing coated spore, and as the ampty heads of those adsorbed phages implied, DNA of SP816 might have penetrated into these sporulating cells. Thus the arrest of phage multiplication seemed to be mediated by intracellular events. Losick et al. (1970) have demonstrated that during sporulation, one of the β-subunits of the RNA polymerase was modified and replaced by a polypeptide with a lower molecular weight, and that the vegetative sigma factor was unable to restore the activity of the sporulating core enzyme with ϕe DNA as template. And they concluded that the loss of stimulation capacity of the sporulating sigma factor was due to the modification of the β-subunit in the RNA polymerase of the sporulating cells, and the change in template specificity of the RNA polymerase, in turn, was responsible for the arrest of the multiplication of the phage ϕe in the sporulating cells. Since the multiplication of SP816 was blocked at early stage of sporulation, the RNA polymerase in *B. subtilis* SNU816 may also be changed during sporulation. However, as was reported by Kawamura and Ito (1974) who showed that transcription of phage DNA occurred *in vivo* well after the occurrence of host changes that inhibit phage replication, and suggested that the changes in template specificity of host RNA polymerase probably could not account for the inability of phage to multiply at early stages of sporulation, the arrest of phage multiplication in sporulating cells may

possibly be caused by more complex reasons. Therefore it leaves more detailed researchs to be performed to visualize the real causes of arrest of phase multiplication in sporulating cells.

적 요

Bacillus subtilis SNU 816의 SP816 박테리오파아지에 대한 감수성의 변화를 조사하였다.

이 균은 영양형성장 중일 때에는 파아지에 대해 감수성을 나타내었으나 대수기 후반을 지난 후에는 파아지의 감염에 대해 저항성을 보였다. 포자형성이 억제된 상태에서는 감염시간에 관계없이 파아지에 의해 용균이 되었으므로 파아지에 대한 감수성의 변화는 포자형성에 관계될 것으로 보였다. 실제로 파아지의 증식은 포자형성의 초기단계에서 억제되었으며 전자현미경적 관찰에 의해 포자형성 2단계인 septum의 형성 중 혹은 바로 그 전에서 억제되는 것이 확인되었다.

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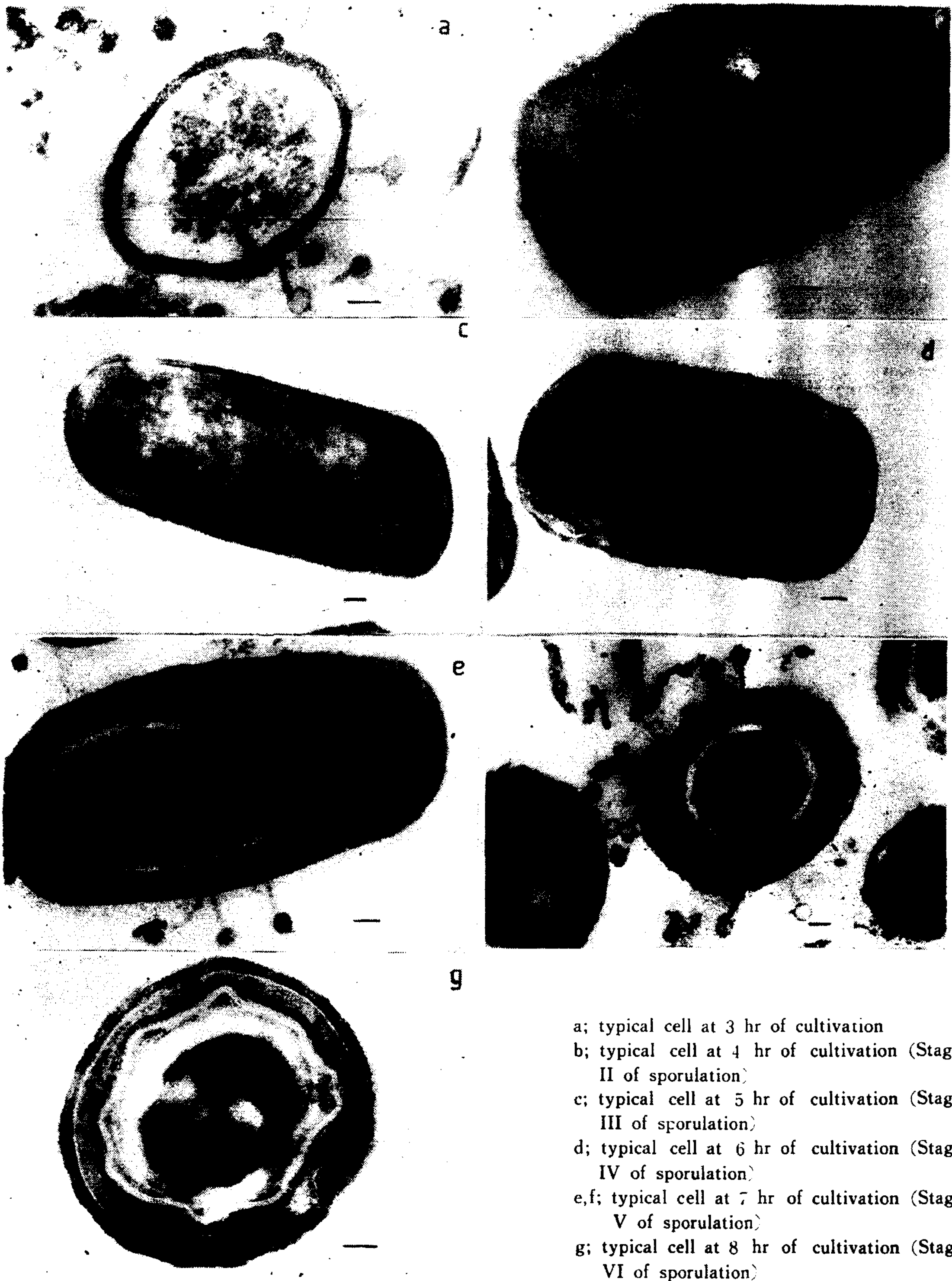

Plate 1. Transmission electron microscopy of sporulating cells of *Bacillus subtilis* SNU816 in-fected with phage SP816. Pictures were taken 50mins after phage infection(see Fig. 3). The length of each bar represents 0.1μm.