BACTERIAL LEAF BLIGHT OF RICE PLANT

II. The formation and properties of spheroplasts of *Xanthomonas oryzae*[1]

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Abstract

The properties of glycine-, penicillin- and lysozyme-induced spheroplasts of two strains of *Xanthomonas oryzae* were investigated. The formation of spheroplast was different between two strains used. Strain 507 was easy to be induced into spheroplasts with various inducers, however, strain 500 was very difficult. The formation of flagella was not affected by the treatment of spheroplast inducers. After treatment the flagella could be easily detected under electron-microscope. Alternation of bacterial surface affected both pathogenicity and phage adsorption. Pathogenicity of lysozyme-spheroplasts, tested on the leaves of rice plant, was remarkedly reduced and phage adsorption completely blocked.

Introduction

Recently, the electron-microscopic studies of thin sections from plants infected with yellow type diseases brought the idea that mycoplasma-like bodies were associated with these diseases (Doi *et al.* 1967; Moramorosch *et al.* 1968; Ploaie, 1968; Shikata *et al.* 1968). Although additional evidences strongly supported the view that yellow type diseases were caused by Mycoplasma (Ploaie and Moramorosch, 1969; Davis *et al.* 1968), however, whether those mycoplasma-like bodies are L-form of bacteria remains to be clarified. Since Mycoplasma and L-form of bacteria are morphologically similar (Weibull *et al.* 1965; Dienes and Bullivant, 1968), and a hypothesis that bacteria proper

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can transform into Mycoplasma via the L-form and that Mycoplasma again via the L-form can convert into bacteria has been suggested (Wittler *et al.* 1956; Smith *et al.* 1957; Kelton *et al.* 1960).

Xanthomonas oryzae causes leaf blight on rice plant. The typical symptom of the disease is characterized by whitish blotches or stripes on rice leaves. However, in some cases where rice plants are severely infected by this pathogen, stunting and chlorosis can be observed at the later stages of disease development. The symptom is systemic and similar to that of yellow type diseases. The cause of the change of disease symptom is still unknown and may be very complicated. One of the possibilities is that the change of symptom might be due to the change of normal rod form of bacteria to L-form in the diseased tissue under a certain environmental condition.

As a preliminary to the study of the conversion of rod-form of bacteria to L-form and relationship between L-form of bacteria and Mycoplasma, the formation of spheroplast of two strains of *X. oryzae* and some properties of the spheroplasts were investigated and reported here.

Materials and Methods

Organisms and culture condition: The bacteria used in this investigation were strains 500 and 507 of *Xanthomonas oryzae.* They were serologically different (Lin *et al.*, 1969). Phage used was XP12 of *X. oryzae* (Kuo *et al.* 1968). The bacteria were maintained on PS agar slants containing potato, 200 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $Na_2HPO_4 \cdot 12H_2O$, 2.0 g; peptone, 5.0 g; sucrose, 15.0 g; agar, 15.0 g and 1 liter of deionized water. For phage adsorption experiment, PS medium containing 0.08 M sucrose was used to prevent the spheroplasts from breaking. The concentration of sucrose higher than 0.2 M in PS medium caused the inhibition of phage adsorption, however, the concentration of sucrose at 0.08 M did not cause measurable effect on the adsorption of XP 12 phage. The cultures used in the experiments were grown in 120 ml Erlenmayer flasks containing 25 ml of liquid medium. The flasks were shaken for 18 hr at 30°C on a rotatory shaker.

Lysozyme spheroplasts: The overnight cultures were harvested by centrifugation at 3,000×g for 10 minutes and subsequently washed once in the centrifuge tube with 0.1 M tris buffer at pH 8.5 and finally resuspended in same tris buffer containing 0.08 M sucrose. To 10 ml of bacterial suspension (bacterial cells 2×10^9/ml), 0.8 ml of 0.11 M EDTA, 0.5 ml of 0.5% of bovine serum albumin and 0.8 ml of lysozyme (2 mg/ml) were added. Two min. later 0.4 ml of 0.5 M $MgSO_4$ was added to stop lysozyme action. The preparation was then examined in a phase contrast microscope.

Glycine spheroplasts: These were obtained by adding 1 ml of an overnight culture to 30 ml liquid medium supplemented with 0.08 M sucrose and various concentration of glycine. The cultures were aerated and samples were taken at various time intervals and examined under electron microscope.

Penicillin spheroplasts: The method for the production of penicillin spheroplasts was similar to that of glycine spheroplasts except glycine was replaced by penicillin.

Phage adsorption study: To test spheroplasts for phage adsorption, strain 507 and its specific phage were employed. Spheroplasts were mixed with phage at an infection multiplicity of 1/10 by using 10^8 bacterial cells per ml in liquid medium containing 0.08 M sucrose. Normal rod form bacteria reacted with same phage was used as control. Ten minutes later 10 ml of samples were removed and centrifuged at 3,000×g for 10 min. The supernatant fluid was saved and the residue resuspended in 10 ml of liquid medium and then precipitated by centrifugation. Same procedure was repeated twice. Each time the supernatant was saved and free phages were checked with Adam's soft agar technique (1959). The final residue was resuspended in 10 ml of liquid medium and assayed for infective centers by the soft agar technique.

Electron-microscopic examination: Spheroplasts and normal bacterial cells were fixed in 0.14% formalin for 24 hr and washed once in saline. A drop containing about 10^7 bacteria per ml was placed on a copper grid coated with a Formvar film and backed with a thin layer of carbon; and the excessive drop was sponged off with filter paper. The specimens were directly shadowed with platinum or stained with 1% uranyl acetate at pH 5.0 and then examined with Hitach Hu-11A electron microscope.

Virulent test: To test the virulence of spheroplasts of *X. oryzae*, four-week old rice plants (Taichung 65) were selected. The penicillin or lysozyme induced spheroplasts were washed with PS medium containing 0.1 M sucrose and finally resuspended in the same medium (1×10^8 spheroplasts per ml). Then they were smeared on the middle of leaf blads. The areas smeared with inoculum were punctured with a needle and then covered overnight with wet cotton to maintain humidity. The normal rod form of *X. oryzae* treated in the same way were used as control. Ten days later the typical symptom could be observed on the middle of leaf blads.

Results

Induction of spheroplasts of X. oryzae:

Spheroplasts of *X. oryzae* could be induced by treating the bacteria with glycine, penicillin or lysozyme. The formation of spheroplasts was remarkably different between these two strains used. Strain 507 was rather easy to be

induced with various inducers, while strain 500 was quite difficult.

As indicated in table 1, when strain 507 was treated with 2 mg/ml lysozyme, 99% of bacterial cell were converted into spherical form in three minutes. Higher concentration of lysozme caused the lysis of bacterial cells. For the optimum formation of spheroplast of strain 500 higher concentration of lysozyme (3 mg/ml) was required, however, the conversion was never more than 4% and was not improved by further increase in the concentration of lysozyme.

The formation of spheroplasts in glycine-containing medium was affected by the concentration of glycine used. As indicated in Table 1, at glycine concentrations of 0.1 to 1.0% the strain 507 were able to grow and divide as spherical forms. The formation of the spheroplast was very slow and it took 40 hours for complete conversion. Higher concentration of glycine inhibited the growth of bacteria. With an inoculum of 10^6 viable cells/ml of strain 507, a viable count of 7.5×10^8 to 1.0×10^9 spheroplast/ml could be obtained after

Table 1. *The formation of spheroplast with two X. oryzae strains in different concentrations of lysozyme, pencicillin or glycine*

Inducers and their concentrations	Bacterial strains	
	500	507
Lysozyme (mg/ml)(1)	*	*
1	1.0	50.0
2	3.0	99.0
3	4.0	100.0
4	—	—
Penicillin (unit/ml)(2)		
100	0.0	98.0
200	0.0	100.0
400	0.0	100.0
Glycine (mg/ml)(3)		
0.1	0.0	0.0
0.5	0.0	1.0
1.0	2.0	5.0
2.0	5.0	20.0
5.0	6.0	98.0
10.0	8.0	100.0

* Percent spheroplast formed.

(1) Lysozyme induced spheroplasts were examined 2 minutes after treatment. The reaction was stoped with $MgSO_4$.

(2) Penicillin induced spheroplasts were examined after 5 hour incubation.

(3) Glycine induced spheroplast were examined after 48 hour incubation.

48-hour incubation at 28°C. The formation of spheroplast of strain 500 were limited in a very low rate, even in higher concentration of glycine and longer incubation time.

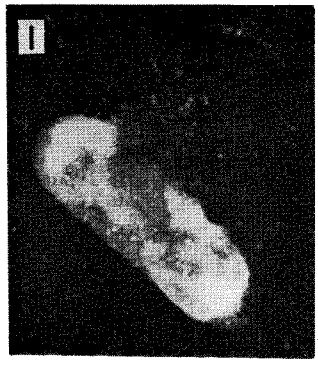
The effect of various penicillin concentrations was similar to that of glycine. However, the formation of spheroplast with penicillin was faster than that with glycine. For strain 507 only five hours was required for complete conversion of rod form to spheroplast at 200 unit per ml of penicillin. Higher concentration of penicillin inhibited bacterial cell division, but the conversion of rod form into spheroplast was 100 percent. No spheroplasts were formed when strian 500 was cultivated in penicillin medium at 500 units per ml. All of the spheroplasts produced by the methods described above were motile.

Morphological observation of spheroplasts:

The morphology of the spheroplasts as viewed by phase-contrast microscope, appeared to be similar irrespective of the induction methods. They were spherical and larger than the normal rod-form. In order to demonstrate more detail about spheroplast, the samples taken from different induction period were made and examined under electron-microscope. Since there was very little difference in morphology attributable to different inducers, only the glycine-induced spheroplasts of *X. oryzae* are shown here.

After 10-hour incubation in glycine medium, the bacterial cells started to show abnormal. Since glycine inhibited the cell wall synthesis a certain particular area of the cell wall became weak. The enlarging of protoplast break the weak area of cell wall and leaks out (Fig. 1). When breakings occurred at the two end of a bacterial cell, owing to the extrusion of protoplasm, the bacterial cell became more oblong or dumb-bell shaped (Fig. 2). When breakings occurred at the middle of one side, extrusion of protoplasm forces the bacterial cell to bend to the other side and results a kidney-shaped cell (Fig. 3). At this stage only 2 to 3% of bacterial cells are completely converted into spheroplasts. After 40-hour incubation 99% of normal bacterial cells converted into spheroplasts. However, a small amount of bacterial cells still remained in incomplete form and some parts of cell wall still attached on the spheroplasts (Fig. 4). It was difficult to determine whether the cell has lost its cell wall when samples were negatively stained with uranyl acetate, but when the samples were prepared with shadowed casting an area of residual cell wall was observed on the spheroplasts (Fig. 5). It appeared that the spheroplasts project from the existing wall. However most of these spheroplasts were completely free from cell wall and attached long flagella (Fig. 6).

Phage adsorption: As described earlier, phage adsorption was determined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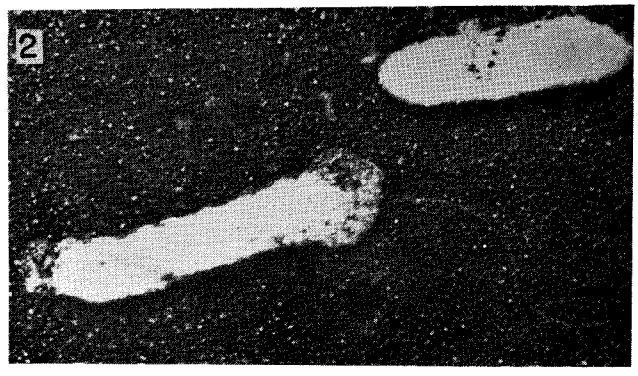
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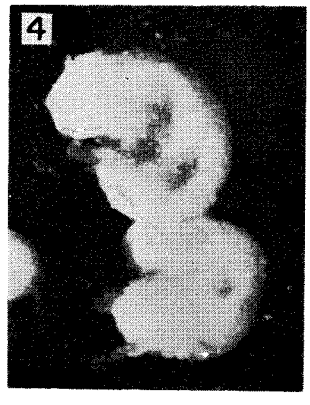
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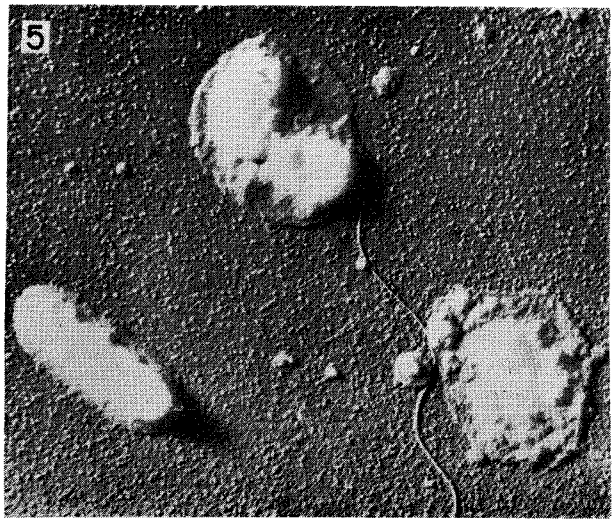
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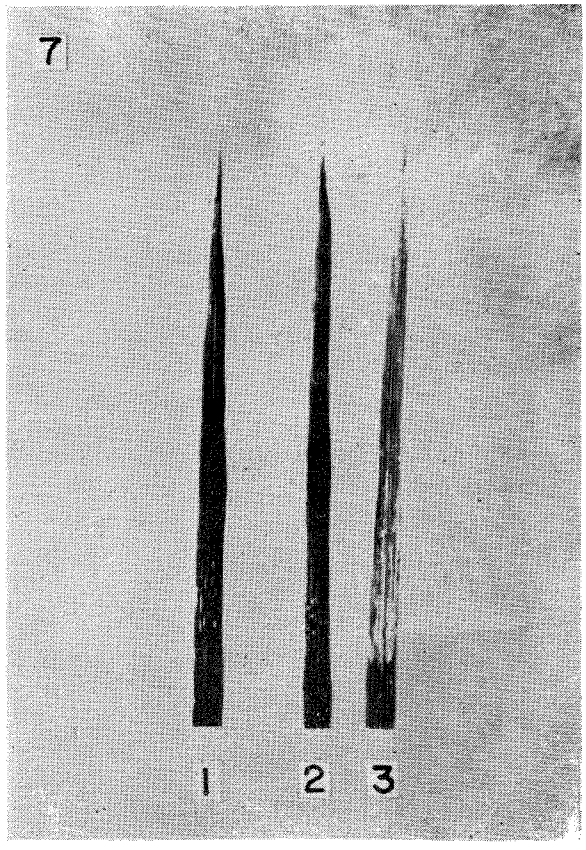

Fig. 1. Protoplasm leak out from the broken cell wall. Uranyl acetate staining (×35,000).

Fig. 2. Spheroplasts extruded from the two end of bacterial cells, the bacterial cells became long and dumb-bell shaped. Uranyl acetate staining (×35,000).

Fig. 3. Breaking occurred at the middle of one side of bacterial cells resulted a kidney-shaped cell. Uranyl acetate staining (×35,000).

Fig. 4. Incomplete form of spheroplasts. Uranyl acetate staining (×35,000).

Fig. 5. Spheroplast with the residual cell wall. Shadowed with plantinum (×28,000).

Fig. 6. Spheroplast with a long flagellum. Shadowed with platinum (28,000).

Fig. 7. Pathogenicity of *X. oryzae* spheroplasts.
(1) inoculated with medium;
(2) inoculated with *X. oryzae* spheroplasts; and
(3) inoculated with normal *X. oryzae*.

by infective center and by the decrease in number of phage in the supernatant. Table 2 shows the results of the phage adsorption on normal *X. oryzae* strain 507 and lysozyme-induced spheroplast made from this strain. When the number of phage remaining in the first supernatant was counted, the amount of phage left in the supernatant obtained from the mixture of spheroplast and phage was much higher than that from the mixture of normal *X. oryzae* and phage. It showed that the alternation of bacterial surface did affect the phage adsorption. After several washes of residual cells with PS medium containing 0.08 M sucrose, the final suspension of residual cells were assayed. The amount of phage in the suspension of the residue of spheroplast was gradually decreased, however, the amount of phage in the suspension of the residue of normal cells was kept constant. This showed that lysozyme-spheroplast did not adsorb phage XP 12.

Table 2. *Phage adsorption of X. oryzae spheroplasts and normal cells*

Hosts	Samples	Phage titer
Spheroplasts	1st supernatant	5×10^{7}*
	2nd supernatant	1×10^{6}
	3rd supernatant	3×10^{5}
	Final residue	5×10^{4}
Normal cells	1st supernatant	5×10^{6}
	2nd supernatant	2×10^{5}
	3rd supernatant	2×10^{4}
	Final residue	4×10^{7}

* Plaque-forming unit per ml.

Pathogenicity of spheroplast: The bacterial cell wall may play an important role on the ability of its attachment and penetration to host cells. Therefore the infection of *X. oryzae* spheroplasts induced with penicillin and lysozyme were inoculated on the leaves of 4 week-old rice plants As indicated in Fig. 7, no symptom was observed on the plants inoculated with *X. oryzae* spheroplasts. However, the typical symptom was showed distinctly on the plants inoculated with normal *X. oryzae.* Therefore the alternation of bacterial cell wall affected the pathogenicity of this organism.

Discussion

These studies show that spheroplast of *X. oryzae* could be induced with penicillin, glycine or lysozyme. However, different strains showed different stability. Induction of L-form of *X. oryzae* was tried, but it was unsuccessful. When spheroplasts were plated on the solid PS medium containg 0.2 M sucrose,

they could not grow. For the growth of the spheroplasts the specific nutrients may be required. The hypothesis that the conversion of rod-form of bacteria to Mycoplsma via L-form is still necessary to be further studied.

The treatment of *X. oryzae* with glycine, penicillin or lysozyme produced a profound alternation on the bacterial cells. These changes affect not only the morphology, but also the sensitivity to phage and virulence as measured by the direct inoculation on host leaves. From a morphological point of view *X. oryzae* spheroplasts differed from normal rod form in being spherical and larger. The size of spheroplasts was very uniform. From the electron micrographs (Fig. 6) and the mobility of spheroplast it seems that the formation of spheroplast did not affect the synthesis of flagella.

Phage adsorption to lysozyme-induced spheroplast was determined by infective center and by the decrease in number of phage in the supernatant. The lack of phage adsorption to *X. oryzae* spheroplasts may be due to the loss of phage receptors existed in cell wall.

Finally, the infection of *X. oryzae* spheroplasts and normal form on the rice leaves were studied. No symptom was detected when *X. oryzae* spheroplasts were inoculated on the rice plants. It seems that the virulence of *X. oryzae* was lost during the transformation of rod-form to spheroplast, however, the lost of virulence may be due to the unstability of spheroplasts on the rice leaves. There is no other plant pathogen being studied with this respect. But with animal pathogens, Freeman (1964) has reported that glycine induced spheroplast of Brucella were more pathogenic for mononuclear phagocytes than normal Brucella. On the contrary Diena *et al.* (1966) found that cytopathogenicity of *Escherichia coli* spheroplasts for tissue culture was reduced and the infection of the monolayers was retarded as compared with the normal bacillary forms.

水稻白葉枯病之研究 II.
病原細菌 *Xanthomonas oryzae* 之 Spheroplast 之形成及其性質

郭宗德 楊晴美 周德源 林英子

水稻白葉枯病之病原細菌 *Xanthomonas oryzae* 能用 glycine, penicillin 及 lysozyme 處理後使其變成 spheroplast。但 spheroplast 之形成依菌系之差別而異。品系

507很容易形成，但500則很不容易形成。品系507在 5 mg/ml 之 glycine 濃度下培養40小時能使桿狀之細菌全部變爲 spheroplast。在 200 unit/ml 之 penicillin 之濃度下則祇需要5小時。如用 lysozyme 則在 2 mg/ml 之濃度下在2分種內使其變成 spheroplast。被處理形成 spheroplast 之菌體，其鞭毛之形成並不受影響。改變細菌細胞壁後其對病原菌之病原性及被其噬菌體感染之性質似乎有所改變。Spheroplast 不受其噬菌體之感染同時失去病原性。

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