Some Structural Observations of *Pseudomonas syringae* pv. *lachrymans* in Affected Cucumber Leaves

Masahiro OZAKI*, Yoshio EHARA* and Susumu YAMANAKA*

尾崎政博*・江原淑夫*・山中 達*：葉組織内でのキュウリ
斑点細菌病菌の構造に関する 2, 3 の観察

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Pseudomonas syringae pv. *lachrymans* (Smith and Bryan 1915) Young, Dye and Wilkie 1978, a causal bacterium of angular leaf spot of cucumber, usually invades the cucumber tissues through stomata¹⁾. The bacteria multiply only in the intercellular spaces without entering inside the host cells¹⁾. Previously we examined cytological studies on the process of lesion formation on cucumber leaves affected by the bacteria using an electron microscope and pointed out that the earliest and most severe abnormalities in the affected cells were found in the plasma membrane and the chloroplasts, and that deterioration of other cell-organelles followed later²⁾. These facts suggested that the bacteria might excrete some phytotoxic substances and affect the cucumber cells from the outside.

In this paper we report on the characteristics of structural changes of the pathogen, especially the occurrence of surface protuberances, during the infection in cucumber tissues.

Cucumber used in this experiment was *Cucumis sativus* L. cv. Yamatosanjyaku, which is susceptible to angular leaf spot. The plants were grown in 5-inch pots in a glasshouse and were inoculated at 3rd leaf stage. The bacterium used was an isolate, No.7414, of *P. syringae* pv. *lachrymans* and grown on Wakimoto's medium³⁾ at 27 C for 48 hr. In preparing inoculum, the bacteria were washed from agar slant with distilled water, centrifuged, resuspended in fresh distilled water, and adjusted photometrically to an absorbance of 0.1 at 620 nm (ca. 10⁸ cells/ml) as described previously⁴⁾. The bacterial suspension was inoculated into cucumber leaves using a spray gun⁴⁾. Inoculated plants were placed in a moist chamber kept at 25 C for 48 hr, and after that they were transferred into a glasshouse.

For electron microscopy rectangular leaf pieces, ranging in size from one to 2 mm², were excised from the inoculated area at intervals of 24 hr from just after inoculation up to the time of necrotic lesion appearance. The tissues were fixed in a 2% osmium tetroxide at 4 C for 4 hr, dehydrated with an ethanol series and propylene oxide, and

* Faculty of Agriculture, Tohoku University, Sendai 980, Japan 東北大学農学部

1) Williams, P. H. and Keen, N. T. (1967). Phytopathology 57: 254-256. 2) Ozaki, M. and Yamanaka, S. (1980). Tohoku Jour. Agric. Res. 31: 141-148. 3) Asuyama, H., Muko, H. and Suzuki, N. (1962). Laboratory Guide for Plant Pathologists. Japan Plant Protection Association, Tokyo, p. 774. 4) Yamanaka, S., Ozaki, M. and Kato, S. (1980). Tohoku Jour. Agric. Res. 30: 135-141.

embedded in Epon 812. As a control, the bacterial colony grown on the medium was also prepared for electron microscopy by the same procedure as described above. Ultrathin sections were cut on a Porter-Blum ultramicrotome with glass knives. The sections were stained with aqueous uranyl acetate for 30 min followed by lead citrate for 2 min at room temperature and examined with a JEM 100 B electron microscope (Japan Electric Optics Laboratory Co. Ltd.).

Fig. 1 shows the fine structure of the bacteria grown on the medium. The central nuclear region within the bacterial cells appeared as an irregularly-shaped lighter area within the surrounding cytoplasm, and in some specimens the area contained strands of material considered to be DNA. Ribosomes were abundant in the cytoplasm. The cell wall of the bacteria was irregularly undulant and sometimes a few small protuberances were observed on the surface.

In the inoculated leaves, a number of small water-soaked spots began to appear in the inoculated sites at 24 hr after inoculation. Before the appearance of the initial symptom, the bacteria in the tissue were sparsely found in the intercellular spaces of inoculated sites, and had essentially the same structure as those cultured on the medium. When the initial water-soaked spots described above appeared, a large number of the bacteria were found throughout the intercellular spaces of the area, moreover numerous protuberances with swollen tips were observed on the surface of the bacterial cells (Figs. 2, 3). The protuberances were about 20 nm in tip diameter with an overall length in the range 30–50 nm.

At 72 hr after inoculation, tips of protuberances on the bacteria were various in size; some of them were enlarged considerably with the range 40–50 nm in diameter (Fig. 4).

At 96 hr after inoculation, the water-soaked lesion areas were beginning to turn brown color, and the bacteria in the tissues were often surrounded by electron-transparent materials similar to capsules though the surface protuberances were still observed (Fig. 5). These may be excreted materials which accumulated outside their walls. Usually the bacterium is noncapsulate, thus it is assumed that in this stage the excretion of the bacteria had been activated, and the excreted materials became visible owing to their being surrounded with electron dense substances which seemed to have been exuded from the affected cells.

At 192 hr after inoculation, expansion of the lesion areas stopped and the areas were dehydrated and turned white or whitish brown color. Thus, the cells in these lesion areas collapsed, shrank and their insides filled with electron dense materials. Most of the bacteria in this stage were deformed in shape like slender rods and the cell inside was occupied with electron dense materials. Accordingly, it was suggested that these bacteria had already died. In addition to these, other bacteria in which nuclear area separated clearly from cytoplasm and contained aggregated DNA fibrils were also found. These bacteria seemed to be in the process of dying (Fig. 6.). On these deformed bacteria observed in this stage the surface protuberances were no longer observed.

In this experiment, it was particularly noticed that the surface protuberances increased remarkably in number on the bacteria multiplying during lesion development. Formation of such protuberances on bacterial cells has been reported on marine *Pseudomonad*^{5,6)},

5) Wiebe, W. J. and Chapman, G. B. (1968). *J. Bacteriol.* 95: 1862–1873. 6) Wiebe, W. J. and Chapman, G. B. (1968). *Ibid.* 95: 1874–1886.

*Escherichia coli*⁷⁾, and only *P. syringae* pv. *phaseolicola* in plant pathogenic bacteria⁸⁾. Bayer⁷⁾ pointed out that the protuberances of *E. coli* were formed by a sudden reduction of the environmental osmotic pressure. Knox *et al.*⁹⁾ reported that a strain of *E. coli* having surface protuberances produced high molecular weight substances, but such a function could not be detected on other strains lacking the structure. Wiebe and Chapman⁵⁾ reported that the formation of the blebs, so-called surface protuberances, might be a phenotypically stable feature in some strains of marine *Pseudomonas* and an inducible phenomenon in others. In the latter case, they produced the blebs only under a high temperature and a high concentration of nutrients⁶⁾. As mentioned above, it seemed that the occurrence and functions of the protuberances differed with bacterial species and environmental conditions during their growth.

The protuberances on *P. syringae* pv. *lachrymans* were similar to those observed on *P. syringae* pv. *phaseolicola*⁸⁾. In this experiment, the protuberances on the bacteria in the tissue in the early stage of infection were very few in number like those on the medium, however, the surface structure increased to a remarkable extent during the symptom development. It is thus speculated that the protuberance formation is closely related to the excreting function of the bacteria. In fact, recently it has been reported that *P. syringae* pv. *lachrymans* produced lipopolysaccharide¹⁰⁾, polysaccharide¹¹⁾ and other substances¹²⁾ which had toxic effect on cucumber leaf tissues. The details of the relationship between the occurrence of the protuberances and bacterial excretion will be elucidated in future study.

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Explanation of Plate

- Fig. 1. Fine structure of the bacteria grown on Wakimoto's medium, showing diffuse nuclear areas and a few surface protuberances (arrows). $\times 36,000$
- Fig. 2. Bacteria in the intercellular space of cucumber tissue at 24 hr after inoculation. A large number of surface protuberances are found on them (arrows). $\times 48,000$
- Fig. 3. High magnification of the surface protuberances on the bacterium at 24 hr after inoculation. $\times 166,000$
- Fig. 4. Bacterium in the intercellular space of cucumber tissue at 96 hr after inoculation. Some protuberances are enlarged (arrow). $\times 25,000$
- Fig. 5. Bacteria in the cucumber tissue at 96 hr after inoculation. The bacteria are surrounded by a translucent material, retaining numerous surface protuberances (arrow). $\times 39,000$
- Fig. 6. Bacteria within a matured lesion area at 192 hr after inoculation. Severely deformed bacteria (D) were found together with bacteria which began to show signs of degeneration. $\times 28,000$

7) Bayer, M. E. (1967). *Ibid.* 93: 1104-1112. 8) Sigee, D. E. and Epton, H. A. S. (1975). *Physiol. Plant Pathol.* 6: 29-34. 9) Knox, K. W., Vesk, M. and Work, E. (1966). *J. Bacteriol.* 92: 1206-1217. 10) Keen, N. T. and Williams, P. H. (1971). *Physiol. Plant Pathol.* 1: 247-264. 11) El-Banoby, F. E. and Rudolph, K. (1979). *Ibid.* 15: 341-349. 12) Shida, T., Mizuno, H., Takanami, K. and Misato, A. (1978). *Ann. Phytopath. Soc. Japan* 44: 59 (Abstr.).