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A Comparison of Fine Structural Features of *Xanthomonas campestris* pv. *malvacearum* Grown *in Vitro* and in Cotton Plant Lines

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ABSTRACT. Ultrastrcture of *Xanthomonas campestris* pv. *malvacearum* Grown in susceptible (Ac 44) and resistant (Im 216) cotton plants was compared with that of the same bacteria grown to late stationary phase in nutrient broth culture. Bacterial cells grown in broth contained many ribosomes throughout the cytoplasm, and DNA strands occupied the central part of the cell. Polyribosomes and osmiophilic vacuoles were present only in actively growing bacteria.

In susceptible cotton plants, *X. campestris* pv. *malvacearum* had similar structural features to broth-grown bacteria. During late stages of disease, capsular coating material developed on those bacteria embedded in a fibrillar material in host intercellular spaces. In resistant plants, bacterial cells developed similar cytoplasmic structure with no polyribosomes or osmiophilic vacuoles and resembled bacteria in late disease stages of susceptible plants.

Bacteria fixed at late stationary phase in broth differed from those fixed from leaf material in late disease stages. Broth grown bacteria exhibited dissolution, coalescence of ribosomes and nuclear material, as well as vacuole development within intact cell membranes. In late diseased cotton leaves, bacterial cells showed cell membrane degeneration.

Xanthomonas campestris pv. *malvacearum* (Smith) Dye (= pv. *malvacearum*) a rod shaped, Gram (−) bacterium, causes bacterial blight in cotton. This bacterium was studied by Brinkerhoff (1970), who indentified eighteen races of the pathogen using standard differentials developed by Hunter *et al.* (1968). According to Essenberg *et al.* (1979) population levels of the bacterium in susceptible cotton plants increase over a longer period of time than is the case in resistant plant leaves. They also reach a higher final level. Results of an ultrastructural study of similarly diseased cotton plants has been previously

published (Al-Mousawi *et al.* 1982). There are few reports of the fine structure of bacteria involved in plant disease. Most ultrastructural studies of bacterial diseases are concerned with changes in diseased host plants. Sigee and Epton (1975) descried the fine structure of *Pseudomonas syringae* pv. *phaseolicola* in resistant and susceptible leaves of French bean. Horino's (1973) study dealt with morphological changes in rice bacterium, *Xanthomonas campestris* pv. *oryzae* treated with the chemical 2-amino-1,3,4- thiadiazole.

In our earlier work (Al-Mousawi *et al.* 1982), we noticed degenerative changes in pv. *malvacearum* cells grown inside susceptible host plant tissue. The present study was initiated to examine these changes and to compare ultrastructure of the bacteria grown in broth culture as well as in resistant cotton leaf tissue.

Materials and Methods

Plant Environment: Two cotton lines were grown in a greenhouse. These lines were Ac 44, a line susceptible to pv. *malvacearum*, and Im 216, a line resistant to the same pathogen (Brinkerhoff 1970, and Brinkerhoff and Verhalen 1976). Average daytime temperature of the greenhouse was 32 ± 3°C, and night temperature was 20 ± 3°C. Daytime mean relative humidity was 59%, while night time relative humidity was 100%. First and second foliage leaves of cotton were sampled 4-5 weeks after planting, at the time they became fully expanded.

Bacterial Culture and Sampling: Race 3 of pv. *malvacearum* was used in this study. The bacteria were maintained on slants of potato-carrot dextrose agar medium at 15°C. At the time of this study, bacterial cells were subcultured in nutrient broth (Difco laboratories, Detroit, MI 48232 USA) (0.8%) and incubated at 30°C on a shaker. Samples grown for 16 hr in the nutrient broth were at log phase as determined by growth curves (Fig. 1) which measured by plate counts, and sub-samples were taken for electron microscopy (EM). Leaves of Ac 44 and Im 216 were vacuum infiltrated at an inoculum level of 10^8 colony forming units/ml. After bacteria were grown in nutrient broth, another two samples were taken for EM at late log (20 hr) and at late stationary phase (45 hr) when viable bacterial cells had declined. Samples for EM were taken from inoculated leaves of Ac 44 every 12 hr for 6 days and from Im 216 at 5 hr and 22 hr post inoculation.

Electron Microscopy: Bacteria and leaf tissue were fixed with 4% glutaraldehyde in phosphate buffer (pH 7.3) at 4°C for 2 hr. They were washed with 0.1 M phosphate buffer, and post-fixed with 2% osmium tetroxide for 4 hr, dehydrated through an ethanol series, and embedded in firm formulation spurr epoxy resin. Centrifugation (1700 g) was used to change fluids of bacterial cultures. Ultrathin sections were cut with a diamond knife on a Sorvall MT-2

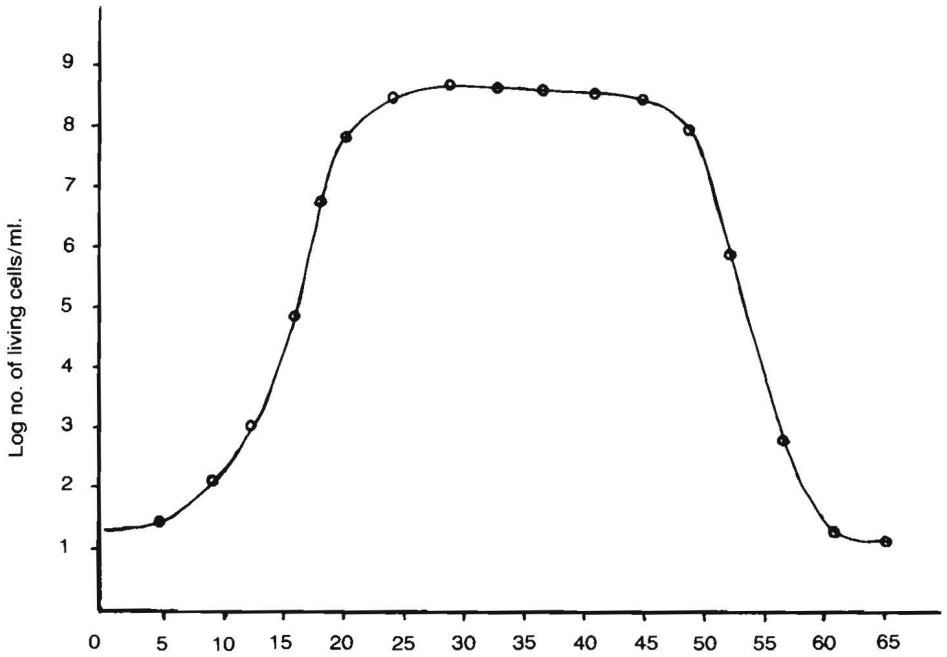

Fig. 1. Population curve of *Xanthomonas campestris* pv. *malvacearum* grown in nutrient broth.

ultramicrotome. Sections were stained 30 min with uranyl acetate and with lead citrate for 3 min and examined at 100 KV with an RCA EMU-3G electron microscope.

Results

Broth grown bacterial at log phase showed the usual wall or multiple cell membranes. Ribosomes of 15-20 nm diameter were distributed throughout the cytoplasm. They were most concentrated in the peripheral part of the cytoplasm (Plate 1). The central portion of the cell was occupied by the nuclear region. Bacteria at log phase had a few polyribosomes about 100 nm in diameter. Osmiophilic vacuoles about 30-50 nm were often found throughout the cytoplasm (Plate 1). At late log phase there were fewer bacterial cells in division, the nuclear regions were smaller, and there were no polyribosomes nor osmiophilic vacuoles (Plate 2). In late stationary phase, a clear zone between cytoplasm and cell membranes developed and ribosomes were distributed evenly within the cytoplasm (Plate 3). Nuclear materials condensed to form dense filaments in the center as well as in the peripheral part of the cells. At this stage the two layers of the outer cell membrane were clearly distinguishable (Plates 3 and 4). Vacuoles or clear zones were also seen inside the cytoplasm. After the above changes

occurred bacterial cell membranes were empty except for a few scattered and lightly stained ribosomes. The two layers of the outer cell membrane were still clear at this stage (Plate 4). Most bacteria had only the outer double membrane with no internal organization, and no cytoplasmic membrane. Cells appeared dead having lost all cytoplasmic components. At this stage the curves from growth studies also indicated no living bacteria.

At twenty-four hr post inoculation, bacteria within susceptible host leaves had similar fine structure to that of cultured bacteria at log phase. Two days post inoculation bacteria were found in the intercellular spaces of the cotton leaves and had increased numbers of polyribosomes. These polyribosomes were concentrated mainly in the polar regions of the bacterial cells. Ribosomes and chromatin fiber distribution was similar to that of log phase broth grown bacteria (Plate 5). At a late stage of the disease (5-6 days) the bacteria were similar to those in stationary phase of broth grown cultures. Fibrillar materials were seen around the bacteria, which were surrounded by clear capsular material 45-80 nm thick. The bacteria had fewer polyribosomes (Plate 6). In some parts of inoculated leaves, dense fibrillar material was seen, and the bacteria were embedded in this material (Plate 7). This occurred at a late stage of the disease when leaves had started to dry, and water-soaked areas characteristic of the disease were present. However, in parts of the dried leaves, bacteria were found in a degenerating condition, with fibrillar material loosened from their cell membranes, and the membranes ruptured unevenly with loss of shape. No capsular material was seen around these degenerating bacterial cells (Plate 8).

In the resistant cotton line, bacterial cells were enveloped 5 hr post-inoculation by material similar to but not as dense as that found later. Bacterial cells had similar cytoplasmic organization to that of bacteria found late in development within susceptible cotton leaves (Plate 8). Fibrillar material was seen inside the envelopments (Plate 9).

Discussion

Ultrastructure of *Xanthomonas campestris* pv. *malvacearum* in these experiments varied with environmental conditions. Polyribosomes and osmiophilic vacuoles were more aboundant in cells grown under favorable conditions and were found in broth grown bacterial cells at log phase and in early diseased cotton leaves. They were not found in broth grown bacteria at late stationary phase, in late diseased leaves, or in resistant plants. In actively growing *Pseudomonas syringae* pv. *phaseolicola* (Sigee and Epton 1975), surface vesicular projections from the bacterial walls were described but were not observed in this study of pv. *malvacearum*. The densely stained osmiophilic vacuoles do not have a known

function, but may contain phospholipids (Grula and Hartsell 1954). Some authors call them inclusions (Wiebe and Chapman 1968) and in some cases they have been shown to contain primarily poly-β-hydroxybutyrate (Martinez 1963).

No capsular or fibrillar materials were observed in nutrient broth grown bacteria, but they were readily demonstrated around bacteria late in the course of the disease. These materials were also seen in resistant cotton leaves at all stages following inoculation. Failure to find such materials in nutrient broth grown bacteria may be due to washing during fixation and embedding or lack of particular nutrients. The capsular material appeared as a clear zone surrounding bacteria. Such material has been shown to stain with ruthenium red when bacterial cultures react with capsule specific-antibody (Bayer and Thurow 1977, and Macki *et al.* 1979).

Fibrillar material around bacteria was seen only in leaves from plants showing disease symptoms and also was seen in resistant leaves within 5 hr post-inoculation. Horino (1976) suggested that fibrillar material in rice vessels, is made by plants as a part of a defense mechanism. However, it has also been suggested that this fibrillar material originates from bacterial cells as exopolysaccharide and it may be formed whenever unfavorable environmental conditions prevail (Al-Mousawi *et al.* 1982). Furthermore, in cotton cotyledons inoculated with dead bacteria, no fibrillar material was seen around enveloped bacteria (Al-Mousawi *et al.* 1983).

In this study there were differences between ultrastructure associated with events which occurred prior to bacterial death in nutrient broth and that of bacteria which grew in susceptible leaves. Bacterial cells in late stationary phase of broth cultures had many features associated with degeneration. These included aggregation of ribosomes and nuclear material, and formation of irregular vacuoles and plasmolysis of the cytoplasm. These stages were followed by disappearance of all organization inside intact cell membranes. In this disease, bacteria are known to remain alive for years inside diseased leaves kept at 4°C. However, even cell membranes of most bacterial cells had degenerated in susceptible diseased leaf tissue by late stages of the disease. Similar results were found by Horino (1973) when *Xanthomonas campestris* pv. *Oryzae* was grown in chemically treated nutrient media or in disease rice plants. In the present study we did not find sporelike structures described by Horino in inhibited pv. *oryzae* (1973).

Plates. 1-4 are Electron micrographs of *Xanthomonas campestris* pv. *malvacearum* grown in nutrient broth culture.

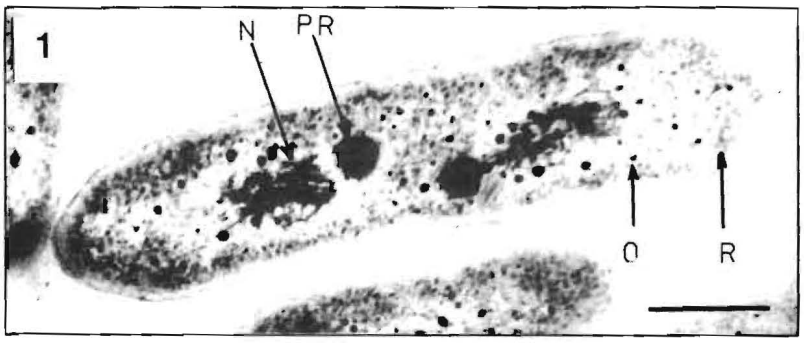

Plate 1. Log phase. Longitudinal view of rod shaped bacterium displaying the cell contents. Nuclear strands (N); Osmiophilic vacuole (O): Polyribosome (PR): Ribosomes (R). The bar represents 200 nm.

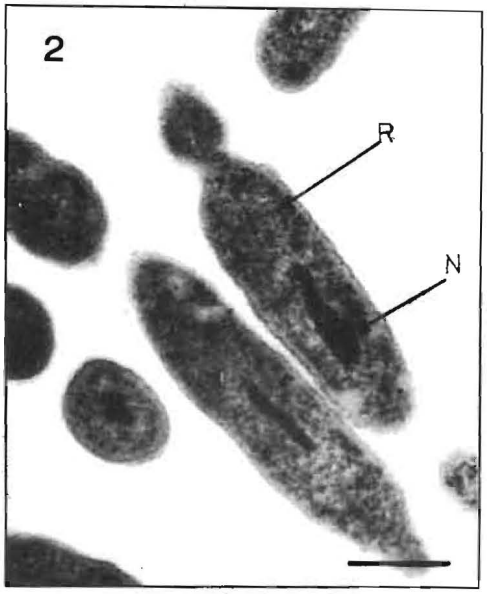

Plate 2. Late log phase, Bacterial cells show condensed nuclar material (N) with aboundant ribosomes (R) and no polyribosomes. The bar represents 200 nm.

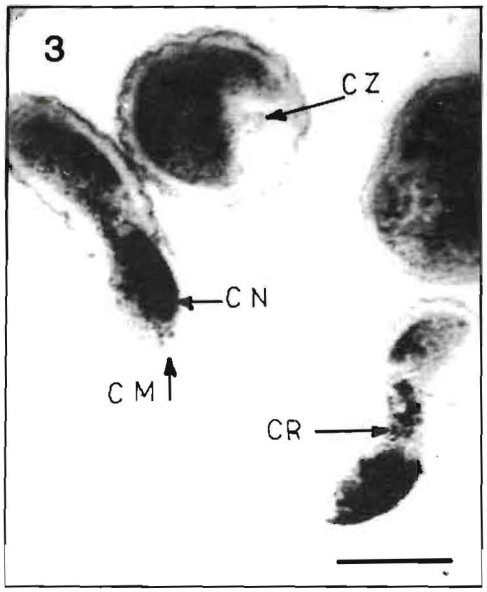

Plate 3. Late stationary phase Bacterial cells showing clear zone (CZ) between cell membranes (CM) and cytoplasm, coagulation of nuclear material (CN) and ribosomes (CR). The bar represents 200 nm.

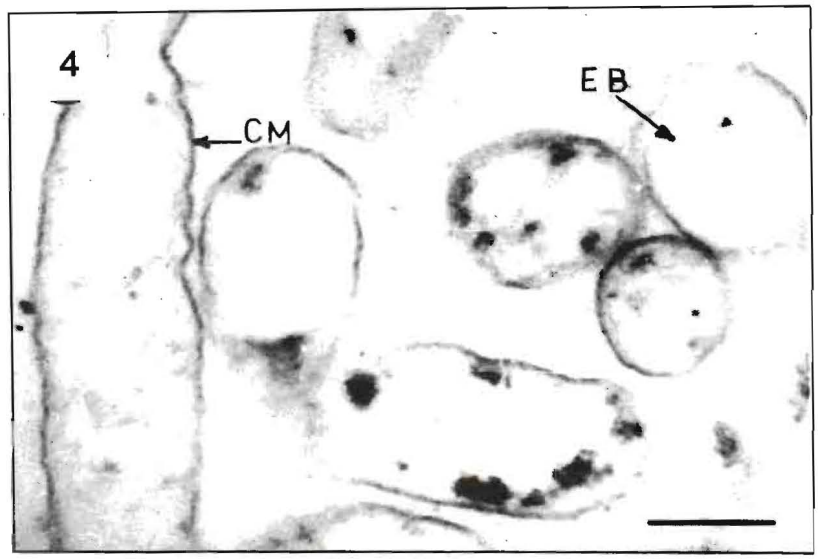

Plate 4. Late stationary phase showing bacteria at different lytic stages. Bacteria look empty (EB) while the cell membranes (CM) appear to be still intact. The bar represents 200 nm.

Plates 5-9 are Electron micrographs of bacterial cells inoculated into cotton leaves.

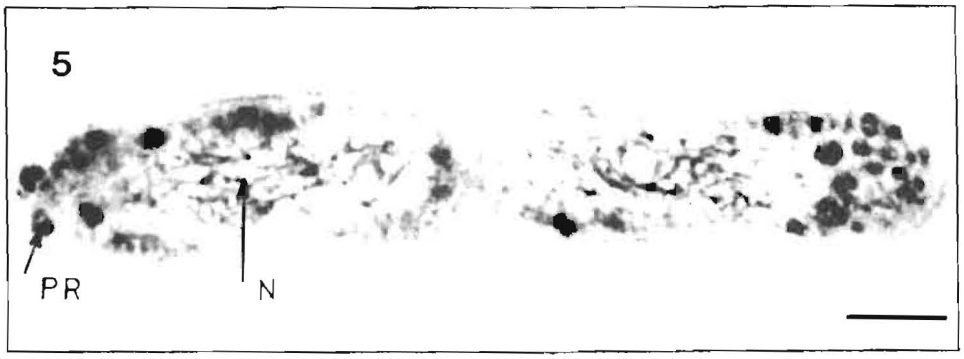

Plate 5. Three days after inoculation into a susceptible cotton leaf, dividing bacterium, many polyribosomes (PR), two nuclear strands regions (N). The bar represents 200 nm.

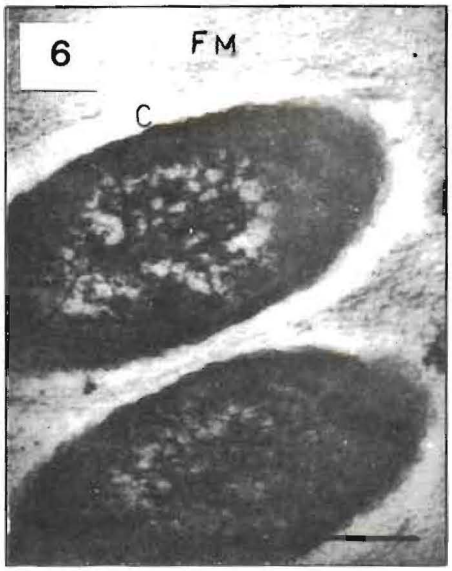

Plate 6. Six days after inoculation into susceptible cotton leaf. Bacterial cells surrounded by clear capsular material (C). The bacteria were embedded in fine fibrillar material (FM). These bacteria were inside a mesophyll cell. The bar represents 200 nm.

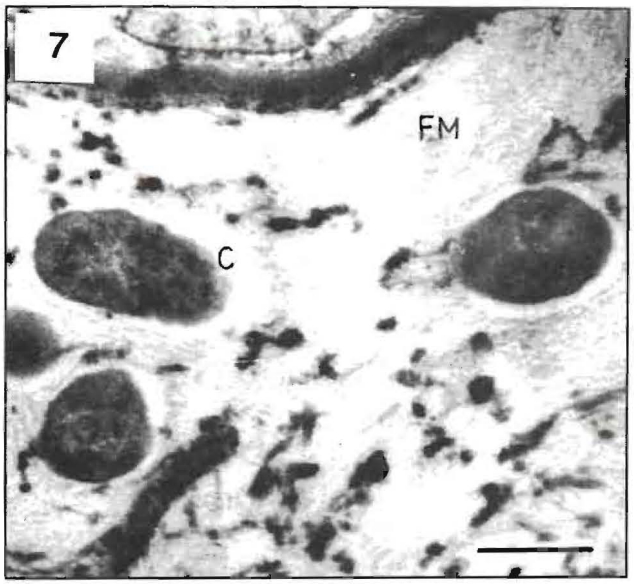

Plate 7. Six days after inoculation into susceptible cotton leaf, bacterial cell embedded in a denser fibrillar material (FM), no polyribosomes and small area occupied by DNA strands. These bacteria were in intercellular spaces of cotton leaf surrounded by capsular material (C). The bar represents 200 nm.

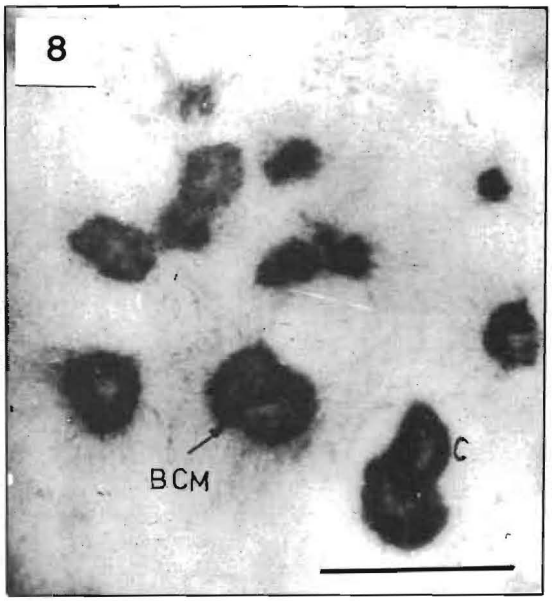

Plate 8. Bacterial cells in different stages of lysis in intercellular spaces of susceptible cotton leaf cells, 6 days after inoculation. No capsular material was seen surrounding lysed cells. Fibrillar material looks loosened from bacterial broken cell membranes (BCM). The lower right bacterial cell looks intact with distinct cell membranes and with many ribosomes embedded in dense cytoplasmic matrix. This bacterium is surrounded by clear capsular material (C). The bar represents 200 nm.

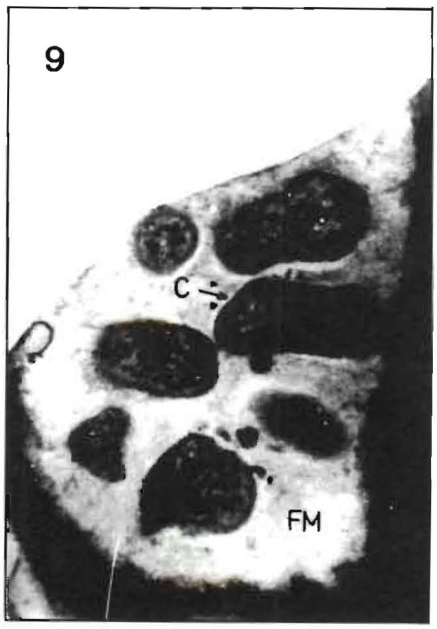

Plate 9. 22 hr after inoculation into a resistant cotton leaf. Bacterial cells are embedded in fibrillar material (FM), and are seen surrounded by capsular materials (C). No polyribosomes are present. Nuclear strands occupied small area. The bar represents 500 nm.

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مقارنة بين المظاهر التركيبية الدقيقة لبكتريا *Xanthomonas campestris* pv. *malvacearum* النامية في الوسط الغذائي وفي ضربين من نبات القطن

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درست التراكيب الدقيقة لبكتريا *Xanthomonas campestris* pv. *malvacearum* النامية في أوراق نباتي القطن غير المقاوم (Ac 44) والمقاوم (Im 216) لهذه البكتريا مقارنة مع نفس البكتريا النامية في وسط غذائي.

تحتوي خلايا البكتريا النامية في الوسط الغذائي على رايبوسومات منتشرة في السايتوبلازم في حين تشغل خيوط DNA وسط الخلية. تلاحظ الرايبوسومات المتجمعة والفجوات المحبة للاوزميوم في الخلايا النشطة للبكتريا فقط.

في النبات غير المقاوم، تشاهد البكتريا وهي تحتوي على نفس مكونات البكتريا النامية في الوسط الغذائي، ولكن في المراحل الأخيرة للمرض، تتكون طبقة كبسولية شفافة تحيط بالبكتريا المطمورة في مادة ليفية في الفراغات البينية لخلايا النبات. ومن جهة أخرى، تلاحظ البكتريا في النبات المقاوم وهي تحتوي على نفس محتويات البكتريا في المراحل الأخيرة في النبات غير المقاوم.

تختلف البكتريا المثبتة في مراحل النمو الأخيرة في الوسط الغذائي عن البكتريا في المراحل الأخيرة للمرض. تظهر البكتريا النامية في الوسط الغذائي اضمحلالاً وتجمعاً للرايبوسومات والمواد النووية كما تتكون فجوة داخل الأغشية السليمه للخليه. بينما في المراحل الأخيرة للمرض، تشاهد البكتريا وقد تحلل غشاؤها.