Motility of *Erwinia amylovora*

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ABSTRACT

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Flagella synthesis by *Erwinia amylovora* is temperature dependent with an optimum at 18–25 C. For motility, a chelating agent and a pH of 6–9 are required. An external energy source is not required for motility although mannitol and glucose are stimulatory. Energy for motility can be derived from oxygen-dependent metabolism of endogenous energy sources. Motility under anaerobic conditions is possible if an energy source that can be metabolized in the absence of oxygen is available. *Erwinia amylovora* cells inside host tissues are not motile but become motile when placed in contact with free water at temperatures optimal for flagella synthesis.

Additional key words: bacterial movement, fire blight, apple.

Most plant pathogenic bacteria are motile by means of flagella. This motility could increase their infection potential by allowing them to reach favorable sites of entry. This has been demonstrated for *Pseudomonas phaseolicola* in which motility increased infectivity (24). Motility in bacteria has been shown to have survival value when nutrients and oxygen become limiting (18,28). Kelman and Hruschka (17) attributed the rapid selection and increase of avirulent over virulent bacteria in broth cultures of *P. solanacearum* to increased motility of the avirulent ones. This phenomenon had been reported earlier in *Salmonella typhimurium* (23) and *P. fluorescens* (28) in which there was a selective multiplication of motile over nonmotile strains.

Environmental factors affecting the motility of *Escherichia coli* and other bacterial species have been examined but such studies have not been done on any plant pathogenic bacterium. This paper describes studies conducted on a peritrichously flagellated plant pathogenic bacterium, *Erwinia amylovora* (Burr.) Winslow et al, the causal agent of fire blight. Motility of *E. amylovora* inside apple shoot tissue also is reported.

MATERIALS AND METHODS

Bacterial strain and media. *Erwinia amylovora* was isolated from a fire blight-infected apple tree shoot. Single-cell isolates were obtained from virulent strains by using the technique of DeVay and Schnathorst (8). Physiological and biochemical tests were done on five of the isolated strains and compared with American Type Culture Collection isolate 19382. A motile strain (A_1) was isolated by the soft tryptone agar motility method (1). Unless otherwise stated, modified Emerson's medium (MEM) (26) was used for growing *E. amylovora* cells and sterile water stock cultures were a source of inoculum for daily experiments.

Factors affecting motility. Effects of growth temperature, pH, chelating agent, energy source, and oxygen on motility were studied by microscopic examination and a motility assay.

Motility was directly observed under a phase-contrast microscope at × 256 magnification by focusing on the bottom of a drop of the bacterial suspension.

Motility assays were conducted according to a slight modification of Adler's technique (2). Basically a capillary tube containing a solution of a chemical to be tested is inserted into a bacterial suspension. If they are attracted to the chemical, bacteria swim to and accumulate in the capillary without a test chemical in the capillary, this technique becomes a motility assay (2). Bacteria move randomly but the number of bacteria in the capillary at the end of the assay period is a function of their rate of movement; the more vigorously motile the cells are, the more bacteria will enter the capillary.

Our modifications of Adler's technique (2) included: using glass petri dishes instead of microscope slides; thorough rinsing with glass-distilled water and sterilization of all materials including 1-μl micropipets (Drummond Scientific Co., Bromall, PA 19008); formation of an assay chamber by laying a U-shaped capillary tube with sealed ends between a glass petri dish and a cover slip. The chamber was filled with 0.2 ml of bacterial suspension in motility medium into which a 1-μl capillary tube containing motility medium and sealed at one end was inserted.

Bacteria used in the assay were prepared from cultures in MEM broth. Cells from MEM plates were transferred to MEM broth and, unless mentioned otherwise, were incubated overnight at 23 C on a shaker bath at 120–140 oscillations per minute. These cultures, which were used to inoculate fresh broth, were allowed to grow to an optical density of 0.2–0.4 at 660 μm. Cultures were harvested by centrifugation for 10 min at 3,400 *g* in a centrifuge at 23–26 C, and resuspended in motility medium to a final concentration of 4×10^7 cells per milliliter. Motility medium consisted of 10^{-3}M or 10^{-4}M ethylenediamine tetraacetic acid (EDTA) in 10^{-2}M phosphate buffer .

The effect of growth temperature on motility was studied by growing cells at temperatures ranging 18–33 C. The cells were prepared as above, examined microscopically and assayed for motility. For estimating the effect of pH, *E. amylovora* was grown on MEM, adjusted to different pH values, and the motility of the cells was determined by microscopic examination. The effect of pH on motility was determined by observing cells in phosphate-buffered medium adjusted to various pH values. The effects of EDTA (a chelating agent) and of different energy sources on motility were studied by adding either EDTA at concentrations ranging $10^{-1} - 10^{-6}$M or energy sources at differing concentrations to the motility medium. The effect of oxygen was determined by examining motility of cells in a drop on a slide with the cover slip either sealed with paraffin or nail polish or left unsealed.

Assays were done at 23 C for 45 min. At the end of the incubation

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period, the capillaries were removed and their exteriors were rinsed with water. The sealed ends were broken and the contents were squirted into MEM broth. Appropriate dilutions were made, plated onto MEM agar, and resulting colonies were counted after 24–48 hr. All assays were replicated three times and number of bacteria per capillary was based on duplicate plate counts. The average percent standard deviation was 21% based on replicate determination of the different treatments in the experiments.

Electron microscopy. Cells grown at 23 and 33 C were centrifuged at 1,000 *g* for 30 min, washed, and resuspended in glass-distilled water. A drop of the suspension was deposited on a 75-μm (200-mesh) polyvinyl formal (formvar)-coated grid and allowed to settle for 30 sec. Excess moisture was absorbed with filter paper and cells were negatively stained with phosphotungstic acid (pH 6.8) for 1 min. Samples were air d ied and electron micrographs were made with a Model JEOL JEM 100 electron microscope.

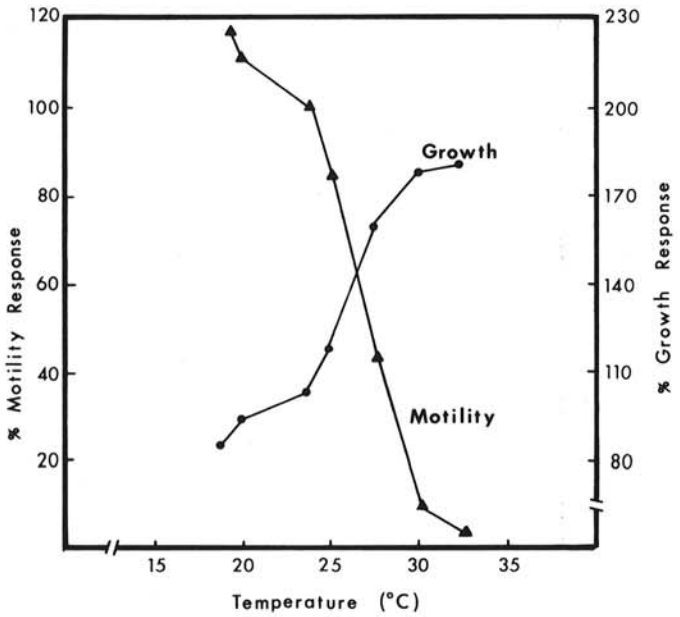

Fig. 1. Effect of incubation temperature on motility (▲) and on growth (●) of *Erwinia amylovora*. The data are a composition of several experiments in which responses are adjusted to 100% with respect to the results obtained at 23 C. Motility assays were performed at 23 C for 45 min with *E. amylovora* suspensions containing 4×10^8 cells/ml suspended in 10^{-4} M EDTA and 10^{-2} M potassium phosphate buffer at pH 7. Growth responses were estimated by dilution plating. Fisher's LSD ($P = 0.05$) = 14.4% for motility and 32.38% for growth.

Motility inside apple shoot tissues. Newly developed succulent apple shoots, 20–23 cm long, (cultivar Jonathan) were cut from mature trees and their cut ends were immediately placed in water. Twenty-four hours prior to inoculation the shoots were sprayed with water, covered with plastic bags, and incubated at either 23 or 33 C. Wounds were made at the base of each newly expanded uppermost leaf to which 16–20 μl of cell suspension (approximately 10^7 cells per shoot) of cultures grown at either 23 or 33 C were deposited. Shoots were sprayed with water, covered with plastic bags, and kept in a lighted incubator at either 23 or 33 C. At 0, 3, 24, 48, 72, and 120 hr after inoculation the topmost 5-mm section was removed and processed as described below. The remaining parts of the shoots were then cut into 5-mm sections which were individually macerated, suspended in 20 μl MEM broth, incubated for 10 min and examined under the microscrope. Five observations were made per section in two trials. The motility index scale of Kelman and Hrushka (17) was used.

RESULTS

Factors affecting motility. *Temperature*. Examination of cultures grown at temperatures of 18–23 C revealed more vigorous cell motility than that of cultures grown at 30 C or higher. Temperature effects on motility were confirmed by Adler's motility assay (Fig. 1). There were no significant differences between responses of cells grown at 18, 20, and 23 C but cells grown at those temperatures were significantly more motile than those grown at 27, 30, or 33 C ($P = 0.01$). At 33 C, less than 1% of the cells were motile and their movements were sluggish. Cells were more vigorously motile when grown below 23 C, but growth rates were not as high as those of bacteria cultured at higher temperatures (Fig. 1).

When cells grown at 23 C were transferred to fresh broth and incubated at 23 or 33 C, only cells incubated at 23 C were vigorously motile after 24 hr. When weakly motile cells (grown at 33 C) were the inoculum, cultures incubated at 23 C became fully motile after five generations (generation time of *E. amylovora* on MEM at 23 C is 81 min). Cells incubated at 33 C remained at a very low level of

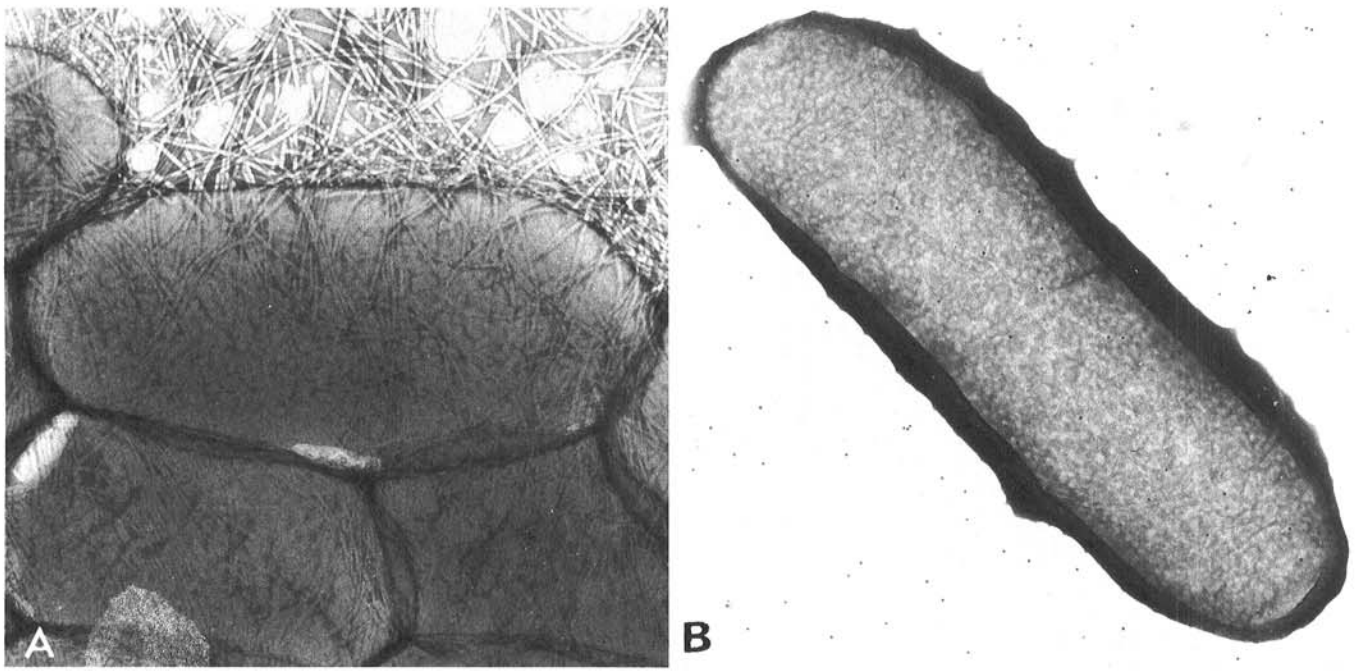

Fig. 2. Electron micrographs of *Erwinia amylovora* cultured at 23 C **(A)** and 33 C **(B)**. (×44,000 and ×84,000, respectively)

motility. When washed cells grown at 23 and 33 C were maintained in motility medium and incubated at either 23 or 33 C, cells grown at 23 C remained motile for about 2 hr while those grown at 33 C, regardless of temperature of incubation, remained only weakly motile. Cells grown at temperatures of 23 C had abundant flagella while cells from cultures grown at 33 C had either very few or no flagella (Fig. 2).

Chelating agents. The addition of EDTA to the motility medium enhances motility of *E. amylovora* as determined by our modification of Adler's method (Fig. 3). Maximum stimulation by EDTA was observed at 10^{-3}M and inhibition at 10^{-2}M or higher concentrations. Motility of cells in phosphate buffer plus 10^{-4}M EDTA was more vigorous than that of cells in buffer or distilled water alone. Addition of other reported chelating agents (2), including 1% peptone, 10^{-3}M L-glutamine, 10^{-3}M L-arginine or a drop of MEM broth enhanced motility.

pH. Active motility was observed in cultures on MEM at pH 6–8, but pH 5 and 9 caused reduced motility and growth. The effect of pH on motility itself was assayed by adjusting the pH of motility medium to different pH values. Motility, as reflected in numbers of cells per capillary, was unaffected within the pH range 6–9 (Fig. 3).

Energy sources. Motility (as determined by microscopic examination) was enhanced when certain energy sources were added to the motility medium. In this experiment the Miller and Schroth medium (20) minus the selective agents was used as the growth medium. Mannitol and glucose at 10^{-3}M gave the best stimulation as compared to cells in motility medium without an outside energy source. Glutamine, asparagine, galactose, fructose, and sucrose at 10^{-3}M also enhanced motility, but not as greatly as did mannitol and glucose. Glycerol at 0.1 or 0.05% or sodium lactate at 10^{-3}M and 10^{-4}M or inositol at 10^{-3}M did not stimulate motility.

Oxygen. Motility of cells (10^7 cells per milliliter) washed with and suspended in motility medium ceased after 30–45 min in a drop on a slide with the cover slip sealed. When the seal was broken, good motility was restored. Cells in the unsealed controls were motile for up to 2–3 hr. With an added energy source, mannitol, in the medium, motility was maintained for 1.5 – 2 hr and for 8 hr in the sealed and unsealed slides, respectively.

Motility of cells inside apple shoot tissues. *Erwinia amylovora* cells observed directly after extraction from naturally infected apple shoots were not motile. Nonmotile cells were observed from macerated 5-mm sections of infected tissues from tip, middle, and advancing portions of the blighted shoots suspended in MEM broth. Bacteria streaked on Crosse and Goodman's selective medium (7) confirmed the identity of *E. amylovora*. If the cut ends of the infected shoots were immersed in water and whole shoots were sprayed with water and covered with plastic bags, motile cells could be observed from the infected shoots after 24 hr. When shoots otherwise similarly treated were not sprayed with water, motile cells were not observed. Nonmotile cells transferred into water did not become motile even after 24 hr; however, those grown in MEM at 23 C did become motile.

The motility rating of cells in artificially infected apple shoots decreased with increasing distance from the point of inoculation (Table 1). The motility rating also was dependent upon the temperature at which the shoots were incubated. After inoculation with cells grown at 23 C (motility rating of 5), the motility of cells on the surface of incubated shoots at 23 C decreased to 4 at 24 hr, to 3 at 48 hr, and remained at that level until 120 hr. The motility rating of bacterial cells incubated in shoots at 33 C decreased to 1 at 24 hr and remained the same throughout the duration of the experiment. Greater numbers of motile bacteria were observed in the lower parts of shoots incubated at 23 C than in those incubated at 33 C, but only to a maximum of 30–35 mm below the point of inoculation for the former and only up to 16–20 mm for the latter. Using cells grown at 33 C (motility rating of 1), motility of cells on the surface of shoots incubated at 23 C increased to 4 at 24 hr, decreased to 3 at

TABLE 1. Motility rating of *Erwinia amylovora* cells from inside apple shoots incubated at either 23 or 33 C

Growth temperature of inoculum (C)	Incubation temperature of inoculated shoot[a] (C)	Post inoculation observation (hr)	Motility index[b]								
			Distance from point of inoculation (mm)								
			0–5	6–10	11–15	16–20	21–25	26–30	30–35	Center[c]	20 above[d] water level
23	23	0	5	–							
		3	5	–							
		24	4	2	1	1	–				
		48	3	2	2	1	1	0	–		
		72	3	3	2	1	1	1	1	0	0
		120	3	2	2	1	1	1	0	0	0
23	33	0	5	–							
		3	5	–							
		24	1	1	0	–					
		48	1	1	1	0	0	0	0	–	
		72	1	1	1	0	0	0	0	0	0
		120	1	1	1	1	0	0	0	0	0
33	23	0	1	–							
		3	1	–							
		24	4	0	0	–					
		48	3	1	1	0	0	0	–		
		72	3	1	0	0	0	0	0	0	0
		120	3	2	1	0	0	0	0	0	0
33	33	0	1	–							
		3	1	–							
		24	1	0	0	–					
		48	1	1	1	0	0	0	0	–	
		72	1	1	1	0	0	0	0	0	0
		120	1	1	1	0	0	0	0	0	0

[a] Succulent apple shoots (20–23 cm long) pruned from mature trees were immediately placed in water. Twenty-four hours prior to and immediately after inoculation, shoots were sprayed with water and covered with plastic bags to maintain high humidity.
[b] Motility index (17) ratings are: 0 = no motile bacteria; 1 = less than 1%; 2 = 1–10%; 3 = 11–50%; 4 = 51–90%; and 5 = 91– 100%.
[c] Five-millimeter section from the center of the shoot.
[d] Five-millimeter section of shoot cut 20 mm above the portion of shoot submerged in water.

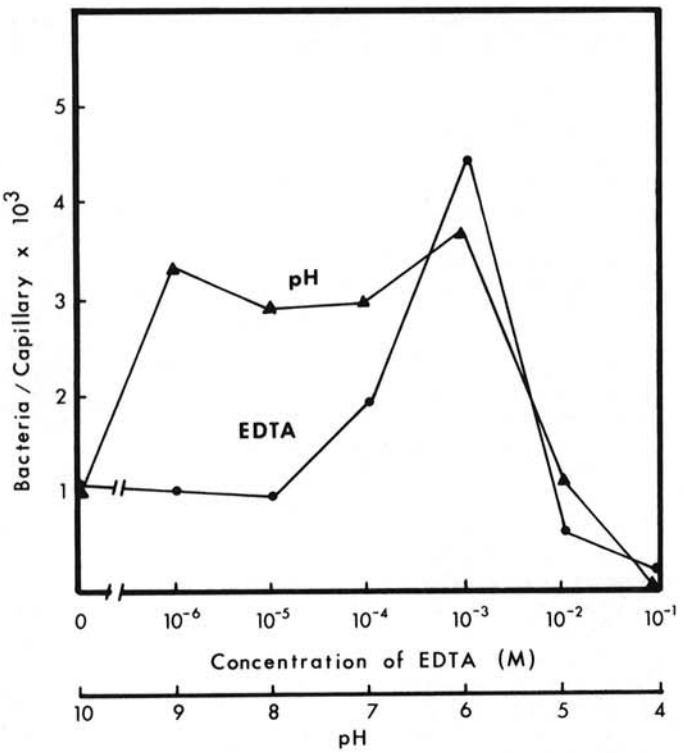

Fig. 3. Effect of ethylene diamine tetraacetic acid (EDTA) concentration (●) and pH (▲) on motility of *Erwinia amylovora*. Assays were performed at 23 C for 45 min with *E. amylovora* suspensions containing 4×10^7 cells per milliliter suspended in 10^{-2} M potassium phosphate buffer for EDTA experiments and in 10^{-2} M potassium phosphate buffer plus 10^{-3} M EDTA for pH experiments. Fisher's LSD ($P = 0.05$) = 525.03 for EDTA and 748.96 for pH.

48 hr and remained at that level through 120 hr. The motility rating of cells grown at 33 C and incubated in shoots at 33 C remained at 1 throughout the experiment. Motile cells were detected to a maximum distance of 16–20 mm but at a low motility rating of 1–2. In all cases at all assay sites, cells in the shoots incubated at 23 C were more vigorously motile than those in shoots incubated at 33 C. Furthermore, few motile cells were found inside host tissues in all cases. The few motile cells were difficult to detect under low power magnification (×256) because they were masked by the nonmotile bacteria and debris. They can be observed, however, under high power magnification (×640).

The inoculated shoots developed typical fire blight symptoms of oozing, discoloration, and wilting at 5–7 days in shoots incubated at both 23 and 33 C.

DISCUSSION

Erwinia amylovora is peritrichously flagellated (5,15). The synthesis of its flagella is dependent upon growth temperature. Flagellar synthesis is inhibited at higher incubation temperatures as reported in *E. coli, Proteus* sp., and *Salmonella* sp. (6,12,21). Electron micrographs revealed that nonfunctional paralyzed flagella are not synthesized at higher temperatures as in the case of some strains of *E. coli* (22). At the optimum temperature (23 C or lower), *E. amylovora* produces abundant flagella. This temperature is not optimal for growth, which in this study was 30 C. Optimum growth temperature for *E. amylovora* is generally between 18 and 30 C (4,11). Differences appear due to the use of different media and variable conditions for growth. Billing (4) showed that growth rates of *E. amylovora* increased linearly in the temperature range 9–18 C and decreased in the range 18–28 C. The temperature cited as minimum for occurrence of blossom blight is 18 C (19,25). This is of interest since motility is optimal at 18 C for *E. amylovora*. It is conceivable that vigorous motility can enhance the infection process, but motility is not an absolute necessity either for entry (9,24) or for pathogenesis because nonmotile *E. amylovora* cells cultured at 33 C are pathogenic.

Environmental conditions and cultural factors influence motility of bacteria. Motility of *E. amylovora* is enhanced by EDTA, possibly because of its chelating ability (3). In *E. coli*, motility was inhibited by trace amounts of heavy metals; EDTA and certain amino acids stimulated motility by chelating metal ions (3). This might explain the restoration of good motility of *E. amylovora* when a drop of MEM broth or amino acid was added to a bacterial suspension washed in distilled water.

Motility of *E. amylovora* can develop without an outside energy source, although a chelating agent is needed to demonstrate this. In *E. coli* this was shown to be due to an endogenous energy source (3). Motility of both *E. amylovora* and *E. coli* (3), however, is stimulated by exogenous energy sources.

Oxygen is required for motility of *E. amylovora* unless an energy source like mannitol, which can be utilized anaerobically, is present. In *E. coli*, oxygen is required for the utilization of energy from endogenous sources (3). However, motility was observed under anaerobic conditions in the presence of serine, an energy source that the bacteria can metabolize in the absence of oxygen. A similar result was obtained with *P. viscosa* (27) which anaerobically utilized arginine as an energy source for motility.

The optimum pH for motility varies with the bacterial species being studied (3,10). The effect of pH on flagella synthesis may not parallel the effect on motility itself. Flagellar synthesis in *E. amylovora* is inhibited at pH 9 as shown by the poorly motile cells resulting from cultures grown on medium at pH 9. However, motility is unaffected at pH 9 since the motility response was identical to that at pH 7. Low pH can cause formation of curly flagella, with corresponding loss of movement as in *Proteus mirabilis* (14), or disintegration of flagellae as in *Salmonella typhimurium* (29). Either observation can explain the inhibitory effect of low pH on motility of *E. amylovora*.

Our results demonstrate that *E. amylovora* is not motile inside host tissues. According to Panopoulos and Schroth (24) motility is hampered in intracellular spaces because of absence of free water. This might explain why nonmotile cells on the surface of artifically or naturally infected apple shoots become motile when allowed to incubate in the presence of free water. However, the temperature has to be optimum for flagella synthesis to occur during the conversion of nonmotile to motile cells.

Nonmotility of cells in host tissues may be due to nonsynthesis of flagella, to production of paralyzed nonfunctional flagella, or to inhibition of motility by certain compounds in the tissues. The last explanation is unlikely since motility was observed in bacteria from the surface of infected apple shoots, even when mixed with macerated host tissues. Attempts to determine whether nonmotility was due to aflagellation or to paralyzed flagellae were made with a scanning electron microscope. The resolution of the microscope was not sufficient to show flagella. Furthermore, preparatory processing of the infected host tissues might have detached the flagellae. Ultrastructural examination of *E. amylovora*-infected tissues by transmission electron microscopy (16) showed bacteria inside host tissues, but no flagellae were discernible. Also no flagellae could be detected in scanning electron micrographs of *Pseudomonas syringae* in *Prunus avium* (13). Perhaps either a preparatory process that would not cause detachment of flagella or a high resolution scanning electron microscope is needed to resolve this question.

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