

LABORATORY TRANSMISSION OF
XANTHOMONAS CAMPESTRIS PV. *RAPHANI* BY A
TARDIGRADE (PARACHELA = MACROBIOTIDAE)

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Tardigrades are animals currently classified in the phylum Tardigrada and are phylogenetically most closely related to the arthropods (Aguinaldo et al. 1997). They live in a variety of microhabitats, being active when a film of moisture is present and becoming dormant when dry. Their small size (mean length 300-350 μ m) allows many of the species to live relatively undisturbed on the surface of a variety of plants and in the soil. The anterior end of the complete digestive system is equipped with sharp stylets and a muscular pharynx which allows a piercing-sucking method of feeding. Some species have a large diameter buccal tube which permits ingestion of solid particles up to the size of fungal spores. Bacteria have often been observed on the surface of tardigrades and are ingested by a number of tardigrade genera (Kinchin 1994). It has been assumed that these bacteria are food, although symbiosis has been suggested (Kinchin 1989, 1993).

During a previous study of lichen-dwelling species of tardigrades, we discovered that up to half of the individual tardigrades examined harbored members of the phytopathogenic bacteria *Xanthomonas* and *Pseudomonas* (Krantz et al. 1999). Evidence developed in that study also suggested that these bacteria dwell inside the tardigrade, perhaps having originated from detritus or food. Furthermore, the bacteria appear to be shed as the animal moves about. Tardigrades harboring phytopathogenic bacteria might transport those bacteria to suitable plant hosts where they could then cause disease. Experimental evidence reported here strengthens this interpretation by showing that in the laboratory tardigrades can acquire and transfer *Xanthomonas campestris* pv. *raphani* Lincoln. Infections can be produced in healthy radish plants from these transferred bacteria.

Xanthomonas campestris pv. *raphani* ATCC 49079 was obtained from The American Type Culture Collection, Rockville, MD. Individual tardigrades, *Macrobiotus hufelandi* Schultze (Parachela: Macrobiotidae), were collected from samples of lichen growing on trees along the shore of a fresh water lake. The samples were soaked in water for 3.5 h during which the dormant forms of the tardigrades became active. The tardigrades were concentrated by filtering the water containing them through 90 or 120 μ m mesh screens. They were immediately collected from the screens, rinsed 3 times in tap water that had been passed through a sterilizing nylon membrane filter, and then used in transmission experiments.

Xanthomonas campestris pv. *raphani* was grown in TGCP broth (per liter: yeast extract, 2.5 gm; glucose, 20 gm; peptone, 2.5 gm; NaCl, 1 gm; K_2HPO_4 , 1 gm; $MgSO_4 \cdot 7H_2O$, 0.5 gm; $CaCO_3$, 10 gm) or on nutrient agar (Difco, Sparks, MD) at 30°C. Radish, *Raphanus sativus* L. ('Cherry Blossom' seed BWI Bulk Seeds, Inc., U.S.A.), were washed with 70% (v:v) isopropanol before planting in sterile 30 $\times$ 290 mm tubes filled to 1/3 of their volume with moist, sterile potting soil and fitted with sterile sponge plugs. The tubes were incubated at 30°C in growth chambers on a continuous 24 h light cycle.

Tardigrades were inoculated with *X. campestris* pv. *raphani* by adding bacteria from a 24 h-old culture to a concentration of approximately 1×10^6 cells / ml of water in which some lichen samples were soaked for 3.5 hours. Confirmation of inoculation was obtained by previously described methods (Krantz et al. 1999) as follows. Tardi-

grades were rinsed 3 times in 50 µl droplets of sterile tap water by using a small wire (Irwin) loop to capture the tardigrades and transfer them from droplet to droplet. They then were placed on the surface of a nutrient agar plate, in the center of a circle (2 cm in diameter) which was marked on the undersurface of the petri dish. The plates were incubated for 5 days at 30°C during which the tardigrades freely moved across the surface of the agar. Microorganisms shed by the tardigrades developed into macroscopic colonies during this period. Smooth, round, lemon-yellow colonies typical of *X. campestris* pv. *raphani* which arose completely outside of the circle were used to inoculate leaves of radish seedlings by first dispersing a single colony in 5 ml of sterile water and subsequently placing 50 µL of this suspension on the leaf of a radish plant. Isolates which produced leaf spot within 7 days were scored as being *X. campestris* pv. *raphani* originating from inoculation of the tardigrade during soaking of the lichens. Uninoculated tardigrades were tested this way also to determine if *X. campestris* pv. *raphani* was associated naturally with the tardigrade population.

To determine if *M. hufelandi* could be inoculated with *X. campestris* pv. *raphani* from the lesions on infected radish leaves, single infected leaves were removed aseptically from separate plants and placed topside-up on separate nutrient agar plates. A single rinsed tardigrade suspended in 50 µl of sterile water was deposited with a micropipet onto the leaf spot lesion of each leaf. The plates were incubated for 48 h at 30°C and scored for the presence of *X. campestris* pv. *raphani* colonies on the agar away from the leaf. The presence of these colonies would indicate that the bacteria had been transferred off the leaf by the tardigrade. As controls, infected leaves without tardigrades and uninfected leaves with tardigrades also were tested.

To test whether tardigrades harboring *X. campestris* pv. *raphani* could facilitate an infection on healthy radish plants, 25 individual radish seedlings were inoculated either with a single inoculated or uninoculated tardigrade prepared as described above. The plants were incubated as described above for up to two weeks and were scored for the appearance of bacterial leaf spot on the leaf where the tardigrade had been placed.

Every attempt to inoculate *M. hufelandi* with *X. campestris* pv. *raphani* was successful. In the 5 replicate experiments in which tardigrades were suspended with these bacteria in water, and subsequently rinsed and placed on nutrient agar plates, 17 to 23 suspected colonies of *X. campestris* pv. *raphani* arose per plate. All of these colonies were used successfully to produce leaf spot disease in healthy radish plants, confirming that they represented the pathovar used to inoculate the tardigrades and not naturally-occurring microbiota. Tardigrades which were not inoculated with *X. campestris* pv. *raphani* did not produce colonies capable of causing disease in radish plants. Plates inoculated with the last drops of sterile water used to rinse the tardigrades produced no colonies.

In all 5 replicates in which uninoculated tardigrades were introduced onto diseased radish leaves lying on the surface of nutrient agar plates, and subsequently were allowed to move freely across the surfaces of the plates, 15 to 24 colonies of the pathogen arose per plate, indicating that tardigrades can acquire and move *X. campestris* pv. *raphani* from diseased leaves. Plates in similar experiments using uninfected, non-diseased leaves produced no colonies of *X. campestris* pv. *raphani* nor did plates containing only diseased leaves without tardigrades.

In all 10 replicates in which inoculated tardigrades were introduced onto the leaves of healthy radish plants, the radish plants displayed symptoms of leaf spot disease within 2 weeks. The symptoms were identical to those observed when a washed pure suspension of *X. campestris* pv. *raphani* only was used to inoculate radish leaves. Inoculated tardigrades therefore were able to shed viable *X. campestris* pv. *raphani* which then

caused an infection on the leaves. Leaves treated with uninoculated tardigrades or cell-free spent TCGP broth from *X. campestris* pv. *raphani* cultures did not become diseased.

Tardigrades were easily inoculated with *X. campestris* pv. *raphani*. No failures to inoculate were observed in the experiments. Although it is uncertain where the inoculated bacterial cells resided, either in or on the animal, they appeared to associate quickly and tightly with tardigrades. The xanthan exopolysaccharide produced by *X. campestris* pv. *raphani* may have mediated attachment of the bacteria to the tardigrade exoskeleton. Or, the tardigrades may have ingested the bacteria while changing from the dormant to active form during the soaking period. Because the tardigrades were not washed free of the attached *X. campestris* pv. *raphani* it is possible that a specialized relationship exists between them. Even when tardigrades were placed on the lesions of infected leaves, the pathogen associated with the tardigrades at least well enough to be transported off of the leaves and across the surface of nutrient agar plates. Perhaps in nature tardigrades can transport *Xanthomonas* spp. through the environment, and perhaps even from plant-to-plant, since tardigrades are mobile and their small size also would allow them to become airborne. *Xanthomonas* spp. have been reported to travel through the environment and possibly from plant-to-plant by a wide variety of means including insects and small airborne particles (Bashan 1985).

The experiments in which inoculated tardigrades were placed on uninfected plants showed that tardigrades can deposit *Xanthomonas* on a susceptible host, which then can become infected. It is unknown whether the pathogen was injected into the leaves during feeding by the tardigrades or merely deposited on the surface during other activities, or some combination of the two. However, every plant that received a single, infected tardigrade developed bacterial leaf spot, indicating that the bacteria remained viable and infectious during their association with the tardigrades and that the animals effectively transported the pathogens. The results as a whole suggest that some cases of *X. campestris* infection in nature may be produced by bacterial cells shed from tardigrades.

SUMMARY

Tardigrades were inoculated with *X. campestris* pv. *raphani* by soaking in a suspension of the bacteria or by contact with leaf spot lesions. Bacteria shed from these tardigrades caused leaf spot disease in radish plants.

REFERENCES CITED

- AGUINALDO, ANNA MARIE A., JAMES M. TUBERVILLE, LAWERNCE S. LINFORD, MARIA C. RIVERA, JAMES R. GAREY, RUDOLF A. RAFF, AND JAMES A. LAKE. 1997. Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature* 387: 489-493.
- BASHAN, Y. 1985. Field dispersal of *Pseudomonas syringae* pv. *tomato*, *Xanthomonas campestris* pv. *vesicatoria*, and *Alternaria macrospora* by animals, people, birds, insects, mites, agricultural tools, aircraft, soil particles, and water sources. *Canadian J. Bot.* 64: 276-281.
- KINCHIN, I. M. 1989. *Hypsibius anomalus* Ramazzotti (Tardigrada) from gutter sediment. *Microscopy* 36: 240-244.
- KINCHIN, I. M. 1993. An observation on the body cavity cells of *Ramazzottius* (Hypsibiidae, Eutardigrada). *Quekett J. Microscopy* 37: 52-55.
- KINCHIN, I. M. 1994. The biology of tardigrades. Portland Press, London. 186 pp.
- KRANTZ, S. L., T. G. BENOIT, AND C. W. BEASLEY. 1999. Phytopathogenic bacteria associated with Tardigrada. *Zool. Anz.* 238: pp. 259-260.