Spread of Plant Pathogenic Bacteria with Fungal Hyphae

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ABSTRACT

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Plant pathogenic bacteria spread along hyphae on agar, the extent depending on the bacterium-fungus combination and nutrients in the medium. Spread near *Pythium ultimum* was better than near *Rhizopus stolonifer* and *Trichoderma koningii*. Cells of *Pseudomonas syringae* pv. *lachrymans* were in static masses adjacent to old hyphae of *P. ultimum*, motile in narrow water bands adjacent to middle-aged hyphae, and not detected near young hyphae. *P. ultimum* was not inhibited by any of five bacterial pathogens. Growth of *R. stolonifer* and *T. koningii* was inhibited by some bacteria, and young hyphae of *R. stolonifer* "avoided" bacteria on agar surfaces by growing under them. Bacteria spread via hyphae 2–3 mm across an air gap and 7.5 mm on a glass surface. Results are discussed in relation to the ecology of plant pathogenic bacteria and to fungus culture contamination by bacteria.

Additional key words: *Agrobacterium tumefaciens*, bacterial motility, *Erwinia carotovora* pv. *carotovora*, *Pseudomonas syringae* pv. *glycinea*, and *Xanthomonas campestris* pv. *campestris*.

The association of bacteria with fungal hyphae and reproductive structures in soil and on agar has been observed for many years and has stimulated much speculation on its nature and significance (3,12,14,18). My attention was drawn to the subject in studies of the movement of plant pathogenic bacteria in association with seedling roots (8). In one test, hyphae of a contaminating fungus, *Rhizopus stolonifer* (Ehr. ex Fr.) Lind, were demonstrated to have spread *Pseudomonas syringae* pv. *lachrymans* (*Psl*) on water agar 8 cm from its initial inoculum location. Spread was independent of that which took place near roots. The phenomenon was so striking that it seemed worthy of more detailed study with *Psl* and other plant pathogenic bacteria.

An examination of literature shows that the spread of plant pathogenic bacteria with fungi has received little attention. In 1920, Smith (15) wrote that work was necessary to determine the extent to which fungi "function as carriers" of parasitic bacteria. Years later, Yarwood's (19) results with bean rust suggested to me that rust fungus hyphae could have spread cells of *P. syringae* pv. *phaseolicola* into wounds made by hyphae. The studies of Louie and Yarwood (11) with a rust fungus and unidentified bacterial pathogens of snapdragon indicated a similar "vector nature" of the fungus, as did Stanghellini's (16) report of the "activation" of soft rot bacteria in potato tubers by a *Fusarium* sp. Plant pathogenic bacteria are readily moved locally by moving water and small

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fauna; and some species are motile, but I am not aware of additional published papers that suggest spread by fungi. Dead, but not live, hyphae appeared to aid the spread of the nonpathogenic bacterium *Bacillus subtilis* in in vitro tests (18).

This paper reports in vitro studies on the spread of plant pathogenic bacteria in association with hyphae of three fast-growing fungi, *Pythium ultimum* Trow, *R. stolonifer*, and *Trichoderma koningii* Oudem. An abstract of part of the work has appeared (7).

MATERIALS AND METHODS

Microorganisms. *R. stolonifer* (isolate TF-1) was obtained as a contaminant from a cucumber seed as described in the first paragraph and was identified by R. Ritter. *P. ultimum* (ATCC 11123) was obtained from A. F. Schmitthenner. *T. koningii* (isolate NS-42) came from a maple branch stub (5) and was identified by G. Kuter. All grew rapidly on water agar. Inocula were 1 mm^3 pieces of agar and mycelium taken from the surface of water agar stock cultures that were stored at 5 C.

Plant pathogenic bacteria were preserved on silica gel (9), revived for stock cultures on medium M66 (see below), and stored at 5 C. Inoculum was prepared by adding 5 ml of water to a tube culture of M66 that had been incubated 7–10 days at 24 C, shaking vigorously, and decanting into 95 ml of water. This yielded about 5×10^7 colony-forming units of *Psl* per milliliter.

Media. Detection media in petri dishes, used mostly for replica printing (see below), were M66 (Bacto nutrient agar, 23 g/L and sucrose, 10 g/L) for bacteria and fungi, and M66A (M66 plus 50 mg of cycloheximide per liter) for bacteria. Occasionally, M71 (4), a medium containing boric acid, was used to detect *Psl* and *P. syringae* pv. *glycinea* (*Psg*). This medium also contained the fungicide cycloheximide. Water agar contained 17 g of Bacto agar per liter. A nutrient agar, M81, was used for nutrient studies. It contained 2 g of Bacto nutrient broth and 17 g of agar per liter.

Dishes were sealed with tape to reduce evaporative water loss.

Enclosure studies. To determine if the location of a bacterium was altered by contact with growing hyphae, a fungus was center-seeded in a petri dish, and bacterial cells were seeded at the same time in a semicircular band 1.6 cm from the fungus (Fig. 1, band seeding). Bands 5 mm wide with a 90-degree opening were made with a velvet-covered replica printer. The opening allowed radial growth of a fungus out of the opening without influence by the bacterium, thus providing in one dish an assessment of inhibition of a fungus by a bacterium. In early tests, bacteria were seeded as one streak in a continuous circle about 1.6 cm from the fungus (streak seeding). In a few tests, filter paper pieces, cut the same size as the bands described above, were dipped in the bacterial suspension, drained, and placed on the agar. Dishes were incubated 6–7 days at 24 C. Fungi reached the dish rim in 3–4 days.

Compartmented dish studies. Agar medium was added to each compartment of four-compartmented glass petri dishes. Compartments were formed by flat glass ridges, which were 1.5 mm wide and about 3 mm above the medium. A fungus was point-seeded near the dish rim in one compartment, and a bacterium was seeded in the same compartment as a 2-cm streak, 1.5 cm from the fungus. Data were taken after 6 days, about 3 days after the hyphae had reached the dish rim of all compartments.

Air gap studies. Special petri dishes were constructed to determine if hyphae crossing an air gap also spread bacteria across the gap (Fig. 2). Dish dimensions were 12×12 cm (inside) and 2 cm deep. A "trap plate" suspended inside the dish lid was 9×9 cm. When 125 ml of agar was added, there was a 2–3 mm air gap between the surface of the suspended trap plate and the agar surface. Fungi were center-seeded in depressions in the agar, and bacteria were seeded around the fungus by the band method. A bacterium was considered to have spread across the air gap associated with hyphae when the bacterium was detected on the surface of the trap plate.

Detection of bacteria. A bacterium seeded at one location on agar was detected elsewhere by several methods. Replica printing was used the most. A circular velvet-covered replica printer 14 cm in diameter (illustrated in ref. 8) was pressed momentarily against the surface to be printed and then momentarily against the detection medium in a petri dish. After incubation, the position of colonies on the medium indicated the original position of the bacterium on the surface printed. The threads on the printer were aggregated into about 5,000 tufts; tuft area was about 3% of the surface sampled. For some tests, a surface was printed sequentially to a medium favoring bacteria and inhibiting fungi (M66A or M71) and another favoring both (M66).

Numbers of bacteria were observed to be associated with hyphae on an agar surface with the light microscope (no coverslip was used). Where they were sparse, bacteria were so small that they could not be detected consistently.

In air-gap studies, bacteria on the trap plate surface were stained with carbol-fuchsin before observation.

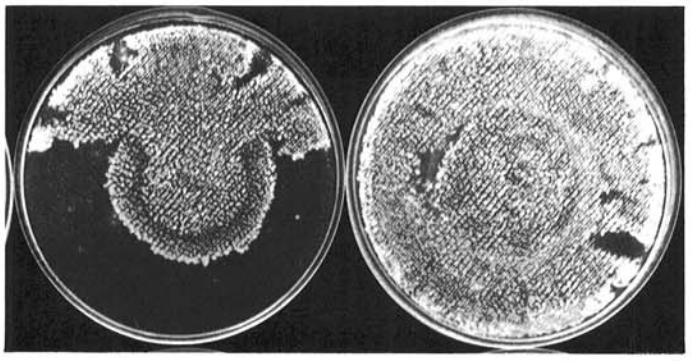

Fig. 1. *Pseudomonas syringae* pv. *lachrymans* (*Psl*) spread with hyphae of *Rhizopus stolonifer* on water agar (left) and on a nutrient agar (right). Photographs are replica prints of test dishes that were center seeded with the fungus and band seeded with *Psl* (the circular open band seen best in the left photograph). The fungus had reached all parts of test dishes in 3 days; prints were made at 7 days. Distribution of *Psl* shown in the replica print is on the detection medium, which contained a fungicide. Pattern on water agar (left) resulted from the "avoidance" of *Psl* by pioneer hyphae of *R. stolonifer* which grew under the *Psl* band and no contact was made. Later, old hyphae within the open band did make contact with *Psl*, which then spread with old hyphae out of the band opening. When nutrients were present, *Psl* spread over the entire test dish (right).

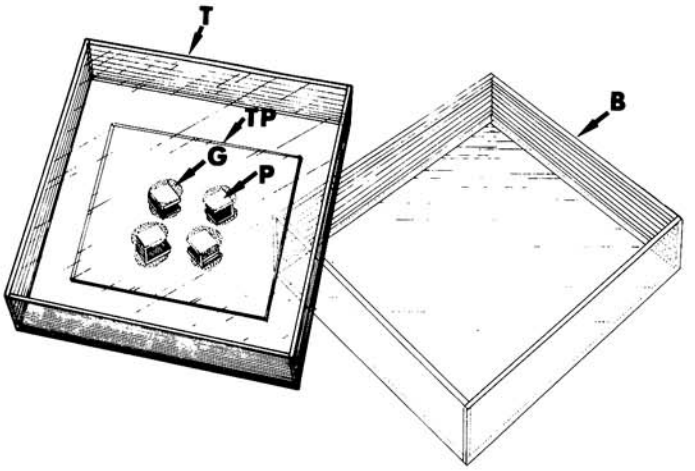

Fig. 2. Petri dish used to determine if bacteria spread with hyphae across an air gap. Dish was constructed of pieces of autoclavable polycarbonate plastic sheet (3 mm thick) fastened together with autoclavable freezer tape (tape not shown). B = dish bottom. T = dish top, shown resting on dish bottom. TP = trap plate, suspended inside the dish top with four aluminum pedicels (P) by means of autoclavable silicone glue (G). Agar was poured into the dish bottom so that when the dish was closed, the surface of the suspended TP was 2–3 mm above the surface of the agar. Bacteria and fungi were seeded on the agar; bacteria on the TP surface were detected by replica printing to an agar medium inhibitory to fungi but permitting growth of bacteria and by staining the TP surface and observing with the microscope.

RESULTS

Enclosure studies with water agar. Table 1 presents a summary of data on the spread of five bacterial pathogens in association with three fungi growing on water agar. Most bacteria spread from their original position with hyphae inwardly toward the fungus inoculum point; fewer were found outwardly toward the dish rim. Except for normal colony expansion, *Erwinia carotovora* pv. *carotovora* (*Ecc*) remained where it was seeded, even though inspection with the microscope showed that hyphae passed among bacterial cells on their way to the dish rim. The other bacteria (Table 1) spread more readily with *P. ultimum* than with *R. stolonifer* and *T. koningii*. Outward movement with *R. stolonifer* often was limited because hyphae of this fungus "avoided" bacterial cells, a type of inhibition described below. Data in Table 1 are from tests utilizing band seeding of bacteria; additional tests with the streak and paper band methods gave comparable results. Thus, the spread of a bacterium with a fungus on water agar depended on the bacterium-fungus combination.

Enclosure studies also demonstrated the inhibition of fungi by bacteria. Some bacteria inhibited *R. stolonifer* and *T. koningii*, but none inhibited *P. ultimum*. Thus, hyphal extension by *R. stolonifer* was reduced 15–25% by *Agrobacterium tumefaciens (At)*, 10–15% by *Psg* and *Psl*, 5–8% by *Xanthomonas campestris* pv. *campestris* (*Xcc*), and not at all by *Ecc*. Moreover, *R. stolonifer* avoided *At*, *Psg*, *Psl*, and *Xcc*, as described below. *T. koningii* was inhibited 5–10% by *At* and *Xcc*; *Ecc*, *Psg*, and *Psl* had no effect on this species. These results are from the same tests noted in Table 1; comparable data resulted from other tests with the streak or paper band methods for inoculating bacteria.

The avoidance of bacteria by hyphae of *R. stolonifer* was studied in some detail with *Psl* by inspecting hyphae with the microscope. There appeared to be no aerial hyphae. As pioneer hyphae grew outwardly on the agar from the inoculum toward the band of bacterial cells, surface growth stopped a few millimeters from the band, and hyphae grew through the agar under the band of bacteria. Hyphae then surfaced a few millimeters outside of the band and continued growing outwardly on the agar surface until the dish rim was reached.

Inward spread with *R. stolonifer* (Table 1, Fig. 1) was associated with older hyphae that grew back into the bacterial cells after the pioneer hyphae had passed under them. These contacts led to the spread of the bacterium inwardly and out of the band opening, as illustrated in Fig. 1. In some tests there was an occasional outward movement of bacteria, because a few older hyphae also grew back into the band. This resulted in the spread of *Psl* in a fanlike manner toward the dish rim. Limited spread of other bacteria avoided by *R. stolonifer* apparently was owing to the same mechanism. It is concluded that on water agar these bacteria in some way inhibited pioneer, but not older, hyphae.

Enclosure studies: effects of nutrients. In three additional tests with water agar and M81 nutrient agar, the interactions between *Psl* and *Xcc* (inoculated by the band method) and *P. ultimum* and *R. stolonifer* were studied. On water agar, the trends shown in Table 1 were confirmed, and inhibition was as described above. On nutrient medium, however, spread of these two bacteria with the hyphae of both fungi was increased and inhibition did not occur. For example, the radial growth and avoidance inhibitions of *R. stolonifer* by *Psl* were not observed (Fig. 1). *Xcc*, which moved in a limited fashion with both fungi on water agar (Table 1), spread to the dish rim on the nutrient medium. Two tests were made with the other bacteria listed in Table 1 with the same conclusions. For example, *Ecc*, which spread with neither fungus on water agar, spread the maximum distance inwardly and nearly the maximum distance outwardly on nutrient medium.

Compartmented dish studies. To determine if *Psl* would spread with hyphae of *P. ultimum* over a glass surface, tests were made with water agar and M81 nutrient agar in compartmented glass petri dishes. Both organisms were seeded in one compartment. In the five dishes containing each medium, *Psl* was isolated from all four compartments and was observed with the microscope to be adjacent to hyphae in each. The bacterium also was observed adjacent to hyphae on the glass ridge tops and was isolated from these areas. The glass surface spanned was about 7.5 mm. There were no aerial hyphae.

Two similar studies were made with *Psl* and *R. stolonifer* on water agar. The fungus was seeded in one compartment and the bacterium in an adjacent one. The bacterium was isolated from the compartment where the fungus was seeded, presumably arriving there with older hyphae after older hyphae made contact with the bacterium in the adjacent compartment. *Psl* also was observed with the microscope to be near hyphae in both compartments and on the ridge top between them.

Air gap studies. Experiments were made to determine if *Psl* or *Xcc* would spread across an air gap of 2–3 mm associated with *P. ultimum*, which was not inhibited by these bacteria, or with *R. stolonifer*, which was inhibited. Results are summarized in Table 2, which shows that both bacteria crossed the air gap with the fungi. Spread with *P. ultimum* was more than with *R. stolonifer*, as could be predicted from inhibition studies. There was more spread with

TABLE 1. Spread of plant pathogenic bacteria in association with fungal hyphae on water agar[a]

Bacterium[b]	Fungus	Bacterial spread (mm) from original position[c]	
		Inward[d]	Outward[e]
At	*Pythium ultimum*	16	23
	Rhizopus stolonifer	16	0
	Trichoderma koningii	10	0
Ecc	*P. ultimum*	0	0
	R. stolonifer	0	0
	T. koningii	0	0
Psg	*P. ultimum*	16	23
	R. stolonifer	16	0
	T. koningii	16	7
Psl	*P. ultimum*	16	23
	R. stolonifer	16	0
	T. koningii	16	7
Xcc	*P. ultimum*	8	7
	R. stolonifer	10	8
	T. koningii	8	0

[a] Fungi were center-seeded on water agar in petri dishes. Bacteria were seeded 1.6 cm away in an opened semicircular band (Fig. 1). Hyphae reached the dish rim in 3–4 days. At 6–7 days the agar surface was replica printed to an agar medium favoring bacteria and inhibiting fungi to determine the position of the bacteria.

[b] *At* = *Agrobacterium tumefaciens*, *Ecc* = *Erwinia carotovora* pv. *carotovora*, *Psg* = *Pseudomonas syringae* pv. *glycinea*, *Psl* = *P. syringae* pv. *lachrymans*, and *Xcc* = *Xanthomonas campestris* pv. *campestris*.

[c] Average of three tests at different times.

[d] Toward the fungus inoculum at the dish center. Maximum distance = 16 mm.

[e] Toward the dish rim. Maximum distance = 23 mm.

TABLE 2. Spread of bacteria across an air gap via fungal hyphae as influenced by the agar medium. Bacterial cells and hyphae were seeded on the agar medium 2–3 mm below a plastic trap plate, which was assayed for bacteria by replica printing

Bacertium[a]	Fungus	Colonies (avg. no.) from the trap plate over:[b]	
		Water agar	Nutrient agar
Psl	Pythium ultimum	357	2,375
	Rhizopus stolonifer	0	10
Xcc	P. ultimum	189	3,070
	R. stolonifer	10	98

[a] *Psl* = *Pseudomonas syringae* pv. *lachrymans* and *Xcc* = *Xanthomonas campestris* pv. *campestris*.

[b] Three tests, except for *Xcc*–*R. stolonifer* (two tests), at different times. Maximum colonies = 5,000.

M81 nutrient agar than with water agar, probably because on nutrient agar there were more hyphae. When the trap plate surface was stained and inspected for bacteria with the microscope, cells of both bacterial species were easily seen in tests with *P. ultimum* and the nutrient agar. Cells were mostly in groups, apparently resulting from growth in condensation droplets seeded by hyphae carrying bacteria. Some hyphae, which apparently had grown along the trap plate surface, had adjacent bacteria similar to those observed on water agar (see below). There was concern that replica printing trap plates would detect bacteria attached to hyphae that were pulled from the agar as the dish was opened. This may have occurred, but the distribution of most stained bacteria indicated that they had multiplied on the trap surface.

Observations of spread of bacteria. The nature of the spread of *Psl* with the hyphae of *P. ultimum* was studied in enclosure tests on water agar. There was maximum movement with this fungus 6–7 days after inoculation (Table 1). When cultures were examined with the microscope after 3–4 days, distribution and activity of *Psl* varied with the age of hyphae. No bacteria were observed associated with young hyphae near the dish rim (replica printing also indicated their absence). In the dish center, near the fungus inoculum, static *Psl* cells were massed in narrow bands on the agar along each side of old hyphae. In the narrow bands of water adjacent to middle-aged hyphae, *Psl* cells swam vigorously. *Psg* also was distributed in the same fashion. When plates were examined 6–7 days after inoculation, when all of the hyphae were old, only static *Psl* cells were adjacent to hyphae. Static *Psl* cells were on each side of old hyphae of *R. stolonifer* and *T. koningii*; hyphae of these species seemed to be degraded by *Psl*, as compared with hyphae with no associated bacteria. Hyphae of *Pythium ultimum* were degraded naturally when sporangia formed, and this did not seem to be accentuated when *Psl* was present.

DISCUSSION

These experiments show that plant pathogenic bacteria spread via fungal hyphae on agar. The degree of movement depended on the bacterium-fungus combination and nutrients in the agar. While these studies were made with plant pathogens, the general findings probably apply to other bacteria as well.

Although they have received little systematic study, observations of bacteria adjacent to lengths of hyphae on agar or glass implies some mechanism of spread. In the present work, bacterial motility (and motility inhibition) in bands of water adjacent to hyphae probably was responsible for the patterns that were observed, and there may also be other mechanisms. Movement may be on the order of centimeters. "Colonization" of mycelia by contaminating bacteria probably accounts for the usefulness of many primary isolation techniques, such as adding bacteria inhibitors to media, and, as the present work suggests, making hyphal tip transfers. Thus, the associations described here may well cause an enormous amount of contamination of fungus cultures, waste, and frustration in microbiological laboratories.

The role of fungal hyphae in the ecology of plant pathogenic bacteria in nature is more speculative. Transport by fungi appears to be of little importance for bacteria on the plant shoot, where, except for bacterial cells that adhere to leaves, pathogen cells can be transported by rain, irrigation water, aerosols, and dew. However, under some circumstances fungal hyphae may provide access to a sheltered wound, and infection court (as work cited above suggests), or to a protected survival site—such as a trichome or bud (6). Where nutrients are sparse, motile plant pathogens probably are attracted to hyphae or propagules, as described for nonpathogenic bacteria (1). Hyphae also probably provide the energy for bacterial multiplication and spread. In the present tests with water agar, there appeared to be no adequate nutrient source other than hyphae for all of the bacterial activity that was observed. Other investigators also have suggested that fungi supply nutrients for the associated bacteria (2,10,12).

Experiments were designed to study situations that may exist in soil. Tests showed that hyphae in a water-saturated atmosphere served to spread bacteria across an air space of 2–3 mm and 7.5 mm on a glass surface. Thus, in soil, the medium so important in the ecology of many plant pathogenic bacteria (13) and so difficult to study (18), hyphae possibly could mediate spread across voids and over mineral faces. This would be expected to depend on the water status of the soil (18), especially as this influences water content of hydrophilic gels produced by hyphae. In addition, spread in natural soil would depend on many complex factors, particularly competition of other microbes (10,17) and the presence of roots and other nutrient sources. In this connection, *P. ultimum* and other oomycetes would be worthy of further investigation. *Pythium ultimum*, a plant pathogen found in many soils, is a pioneer colonizer of plant residues. Four of the five bacteria studied in the present work spread with this fungus on water agar and all were spread with the fungus on a nutrient medium.

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