Pseudomonas morsprunorum, the Cause of Bacterial Canker of Sour Cherry in Michigan, and its Epiphytic Association with *P. syringae*

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ABSTRACT

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More than 462 Gram-negative, oxidase-negative, green fluorescent, and bacilliform bacterial isolates from diseased and from symptomless Montmorency sour cherry buds, blossoms, leaves, fruits, and 1 yr old wood, and new shoot growth were characterized by four determinative tests. Isolates positive for gelatin liquefaction (G) and aesculin hydrolysis (A) but negative for tyrosinase activity (T) and tartrate utilization (Ta) were referred to as GATTa$^+$. Isolates negative for the first two tests and positive for the last two were GATTa$^-$. The GATTa$^+$ isolates utilized lactic acid (L$^+$), hydrolyzed arbutin, and produced a yellow supernatant fluid with a cloudy or translucent appearance in sucrose nutrient broth. All isolates from diseased tissue and about 82% of the isolates from symptomless samples were either GATTa$^+$ or GATTa$^-$. The GATTa$^+$ and GATTa$^-$ isolates were identified as *Pseudomonas syringae* and *P. morsprunorum*, respectively. The hypersensitive reaction on tobacco was not reliable as a criterion for establishing pathogenicity because 15.5% of the isolates induced a reaction on tobacco but failed to infect cherry fruits. Other isolates infected cherry fruits but not tobacco. Syringomycin production also did not correlate well with pathogenicity. *P. morsprunorum*, rather than *P. syringae*, was the predominant species isolated from diseased sour cherry trees. Pathogenic *P. morsprunorum* and *P. syringae* isolates were recovered from symptomless sour cherry tissues in about a 1:1.4 ratio. Epiphytic populations appear to be an important source of inoculum for bacterial canker of sour cherry.

Additional key words: bacterial blast, gummosis, *Prunus cerasus*.

Michigan is the major producer of Amarelle type sour cherries (*Prunus cerasus* L.) in the United States with about 14,000 ha of bearing-age trees of the Montmorency cultivar. Bacterial canker only recently developed as a problem on sweet cherries in Michigan (14) and was not recognized on sour cherries until a severe epidemic in 1976. The sudden outbreak was particularly unexpected because to our knowledge the disease had not been a problem on Montmorency sour cherry.

Symptoms on sour cherry were: necrotic leaf spots with well developed chlorotic halos (Fig. 1A), extensive yellowing and tattering of leaves, severe defoliation, and infected fruits and pedicels exhibited brown soft decay with marginal water soaking (Fig. 1B). Branch and stem cankers (Fig. 1C) were much less common than leaf and fruit infections and were restricted to small areas at the base of infected fruit spurs.

Pseudomonas syringae and *P. morsprunorum* (sensu Bergey's Manual, 7th edition [2]) were identified as the causal agents of bacterial canker of sweet cherry in Michigan (14). Distinction between *P. syringae* and *P. morsprunorum* is based on several physiologic tests: gelatin liquefaction; β-glucosidase and L-tyrosinase activities; utilization of L-leucine, tartrate, lactic acid, D-sorbitol, and i-erythritol as sole carbon sources; growth characteristics in 5% sucrose-nutrient broth; and acid production on purple lactose agar (1,5,10,23,24).

This article describes the isolation, physiologic characterization, and pathogenicity of bacterial populations isolated from diseased and symptomless sour cherry tissue collected in Michigan. The evidence reinforces the phenotypic differences between *P. morsprunorum* and *P. syringae*.

MATERIALS AND METHODS

Sampling sites. In 1976, diseased leaves and fruits were collected from several Montmorency sour cherry orchards in southwest and in west central Michigan. In 1977–1978, samples of diseased and of symptomless tissues were taken from two sour cherry orchards in southwest Michigan and one near Hart in west central Michigan. Infection was severe in these orchards in 1976 but light in 1977 and 1978.

Isolation. Isolations were made on medium B (MB) of King et al (15) containing 50 μg/ml of cycloheximide (cMB). Disease samples were surface-sterilized with 95% ethanol for 1 min and 1% NaOCl for 1 min and then rinsed twice in sterile-distilled water (SDW). Bits of tissue from the margins of lesions were triturated in 1–2 drops of SDW. The material adhering to a glass rod was spread in duplicate on cMB and the plates were incubated at 20 C for 4–5 days.

In 1977 and 1978 pathogenic bacteria were isolated from samples (25 g fresh weight) of symptomless sour cherry buds, blossoms, leaves, fruits (50 g), and branches (1 yr old wood and new shoot growth with leaves removed). Samples were transported to the laboratory in an ice chest and maintained at 5 C until processed.

Tissues were shaken vigorously in 500 ml of SDW for 30–60 sec, diluted in a tenfold series, and 0.1-ml samples were seeded in duplicate on cMB and incubated at 20 C for 4–5 days.

Physiologic and biochemical characterization of bacterial isolates. Bacteria from isolated colonies were first tested for cytochrome oxidase activity (17) and then for green-fluorescent pigmentation on MB and fluorescence under ultraviolet light.

More than 426 bacterial isolates that fluoresced on MB and showed no cytochrome oxidase activity were tested for: (i) gelatin liquefaction (G) by stabbing tubes containing 12% (w/v) gelatin (Difco), which were incubated up to 15 days at 20 C and then held at 5 C for 15 min before determining liquefaction; (ii) β-glucosidase activity (A) on 0.1% (w/v) aesculin agar medium according to Sneath's method (21), except that plates were incubated for 24 hr at 20 C; (iii) tyrosinase activity (T) on agar medium containing 0.1% (w/v) L-tyrosine (19); and (iv) utilization of tartrate (Ta) as a sole carbon source, tested oxidatively on Simmon's basal medium (12) containing 0.2% (w/v) sodium tartrate. Based on the results of

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these four tests, pseudomonads were classified in two groups designated as GATTa$^+$ and GATTa$^-$. The GATTa$^+$ isolates were positive for gelatin liquefaction and β-glucosidase activity but negative for L-tyrosinase activity and utilization of tartrate as a carbon source; GATTa$^-$ isolates were negative for the first two tests and positive for the last two.

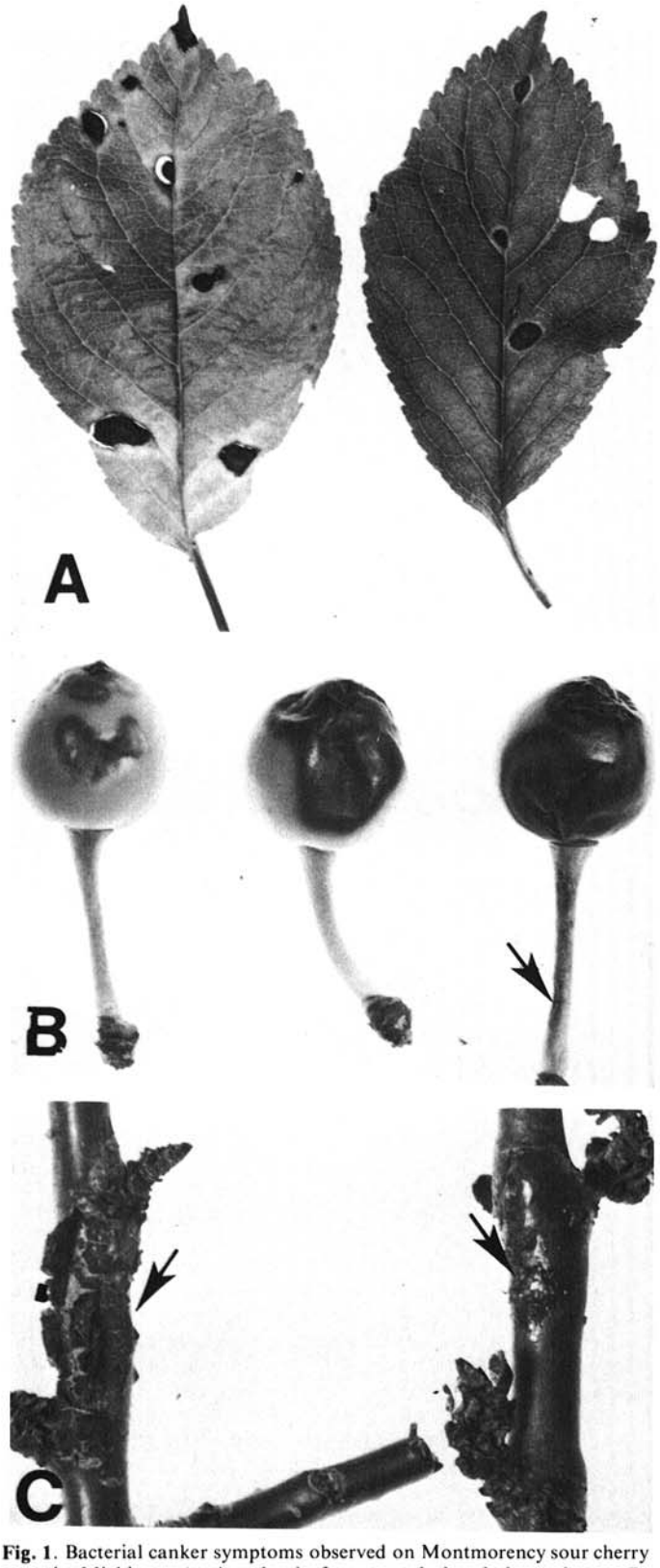

Fig. 1. Bacterial canker symptoms observed on Montmorency sour cherry trees in Michigan. **A**, Angular leaf spots and shot hole on leaves. **B**, Naturally infected fruits with dark brown spots on the fruits and pedicels (arrow). **C**, Stem cankers on 3–4 yr old wood (arrows).

Selected samples of GATTa$^+$ and GATTa$^-$ isolates also were tested for levan production on 5% (w/v) sucrose nutrient agar (Difco) containing 5 ml/L of 1.5% (w/v) alcoholic solution of crystal violet (10) and for β-glucosidase activity on 0.5% (w/v) arbutin-agar medium according to Crosse and Garrett (6) and incubated as long as 7 days at 20 C. Citrate, malic, maleic, and L (+) lactic acid utilization were determined on Simmon's basal medium (12) containing 0.2% (w/v) of the respective organic acid or with L (+) lactic acid at 1% (w/v). D-sorbitol and i-erythritol were tested by stabbing tubes containing 1% (w/v) D-sorbitol or 0.5% (w/v) i-erythritol in a basal medium developed by Hugh and Leifson (13). After inoculation, tubes were covered with 2–3 ml of sterile mineral oil and incubated at 20 C for 3–4 days. Acid production on purple lactose agar (Difco) containing 2% (w/v) lactose and adjusted to pH 7.2 before autoclaving was determined by stabbing or streaking the agar in tubes and incubating them as long as 30 days at 20 C. Growth characteristics in nutrient broth (Difco) supplemented with 5% (w/v) sucrose were determined in 50 ml of broth shaken at 20–23 C for 48 hr. After cultures were centrifuged at 12,000 *g* for 15 min, the color and appearance of the supernatant fluid were observed.

Syringomycin production. Presence of syringomycin was established by the bioassay developed by Gross and DeVay (11). The isolates were grown on potato-dextrose agar (PDA) (Difco) for 48 hr at 20 C before the petri dishes were sprayed with a suspension of *Geotrichum candidum* and incubated for an additional 48 hr. Inhibitory zones of ≥1 mm around the bacterial colonies indicated syringomycin activity.

Reference strains. *P. syringae* isolate P-7-1 was obtained from A. L. Jones, Michigan State University, East Lansing; 5D4214 and B3A were from C. I. Kado and J. E. DeVay, respectively, University of California, Davis; Ch-1-2 was from B. A. Latorre, Universidad de Chile, Santiago; and B160 was from D. J. Weaver, Southeastern Fruit and Tree Nut Research Station, Byron, GA. *P. morsprunorum* isolate Pm-200 was obtained from E. J. Klos and was originally isolated in England by J. E. Crosse. Isolates ATCC 19322, ATCC 13395, and ATCC 13396 were from the American Type Culture Collection, Rockville, MD.

Ice nucleation activity. Isolates were tested for ice nucleation activity as described by Lindow et al (20). Ten 10-μ l drops of bacterial suspension (about 1×10^7 cells/ml) from 24 to 48 hr old cultures on MB were placed on the surface of an aluminum foil boat treated with a 1% (w/v) paraffin xylene solution and floated on a dry ice-ethanol mixture at −4 to −6 C until nucleation of control isolates was observed. Isolates were recorded as positive if at least one drop froze.

Sensitivity to bacteriophage A7. Isolates were tested against phage A7, isolated by J. E. Crosse and Garrett (7) in England and

TABLE 1. Physiologic characterization of Gram-negative and oxidase-negative fluorescent bacteria recovered from symptomless sour cherry tissue in 1977-1978

Source of isolates	Number of isolates	GATTa$^+$[a]		GATTa$^-$[b]		GATTa$^\pm$[c]	
		(no.)	(%)	(no.)	(%)	(no.)	(%)
Buds	60	45	75.0	15	25.0	0	0.0
Flowers	50	27	54.0	11	22.0	12	24.0
Fruits	36	17	47.2	10	27.7	9	25.0
Leaves	150	72	48.0	68	45.4	10	6.6
Branches	130	45	34.6	40	30.8	45	34.6
Totals	426	206		144		76	
%			48.4[d]		33.8[d]		17.8[d]

[a]The GATTa$^+$ isolates are positive for gelatin liquefaction and aesculin hydrolysis but negative for tyrosinase activity and tartrate utilization.
[b]The GATTa$^-$ isolates are negative for gelatin liquefaction and aesculin hydrolysis but positive for tyrosinase activity and tartrate utilization.
[c]The GATTa$^\pm$ isolates are heterogeneous for one or more of the four GATTa tests.
[d]Percentages of the number of isolates per column relative to the total number tested.

supplied to us by E. J. Klos. Samples of the virus suspension incremented on *P. morsprunorum*, Pm 200, were deposited with a platinum loop on the surface of 24–48 hr nutrient agar cultures and incubated for 48 hr at 23 C. The specificity and sensitivity of the virus suspension was established against the above-mentioned reference isolates.

Pathogenicity. The pathogenic potential of 24–48 hr cultures of each isolate was first estimated by its ability to induce hypersensitivity on White Burley tobacco as described by Klement et al (16) and by its ability to infect immature sweet cherry fruits following Jones' procedure (14). Some isolates also were tested on bean pods (cultivar Tender White) according to Ercolani et al (9) and on Montmorency sour cherry fruits, leaves, and shoots. The suspensions were adjusted to OD about 0.15–0.1 at 620 nm in a Bausch & Lomb spectrophotometer and were estimated to contain 1×10^7 cells per milliliter.

Surface-sterilized, immature cherry fruits were inoculated by placing a 10-μl drop of bacterial suspension on each of three punctures made with a sterile needle. Five fruits per isolate were inoculated. Positive results were recorded if at least one fruit developed symptoms after 48–72 hr of incubation in a moist chamber at 20 C. Bean pods were inoculated and incubated similarly.

Sour cherry leaves and green shoots were inoculated by injecting a 1×10^7 cells per milliliter bacterial suspension with a hypodermic syringe. Only the newest four leaves on a shoot were inoculated. Each isolate was injected in three leaves on two shoots. Selected leaves and green shoots were inoculated with about 1×10^7 or with 1×10^5 cells per milliliter. Woody stems were inoculated by delivering a 10-μl drop of a bacterial suspension into a T cut made with a sterile scalpel. Plastic bands were tied around the cuts to avoid rapid dehydration and the plants were held under greenhouse conditions for 1 mo.

RESULTS

Isolation. Oxidase-negative and green-fluorescent bacterial colonies that were consistently isolated from diseased sour cherry leaves and fruits exhibited the following characteristics on cMB: smooth, glistening, and flat colonies with a vitreous appearance against the light; and round to polyhedral shapes with entire or lobate margins. Similar bacterial colonies developed from disease samples of sweet cherry leaves and fruits.

Physiologic characterization. Oxidase-negative and green-fluorescent bacterial colonies similar to those recovered from diseased tissue were isolated consistently from symptomless sour cherry buds, leaves, flowers, fruits, and branches and were separated into three groups based on the GATTa tests (Table 1). Of the 426 isolates characterized, 48.4% were GATTa$^+$, 33.8% were GATTa$^-$, and 17.8% were GATTa$^\pm$. Of the 76 GATTa$^\pm$ isolates, 56 differed from GATTa$^+$ isolates because they were negative for gelatin liquefaction. Without exception, the oxidase negative and fluorescent bacterial colonies isolated from diseased tissue were GATTa$^+$ or GATTa$^-$. Of the 36 isolates from diseased sour cherry

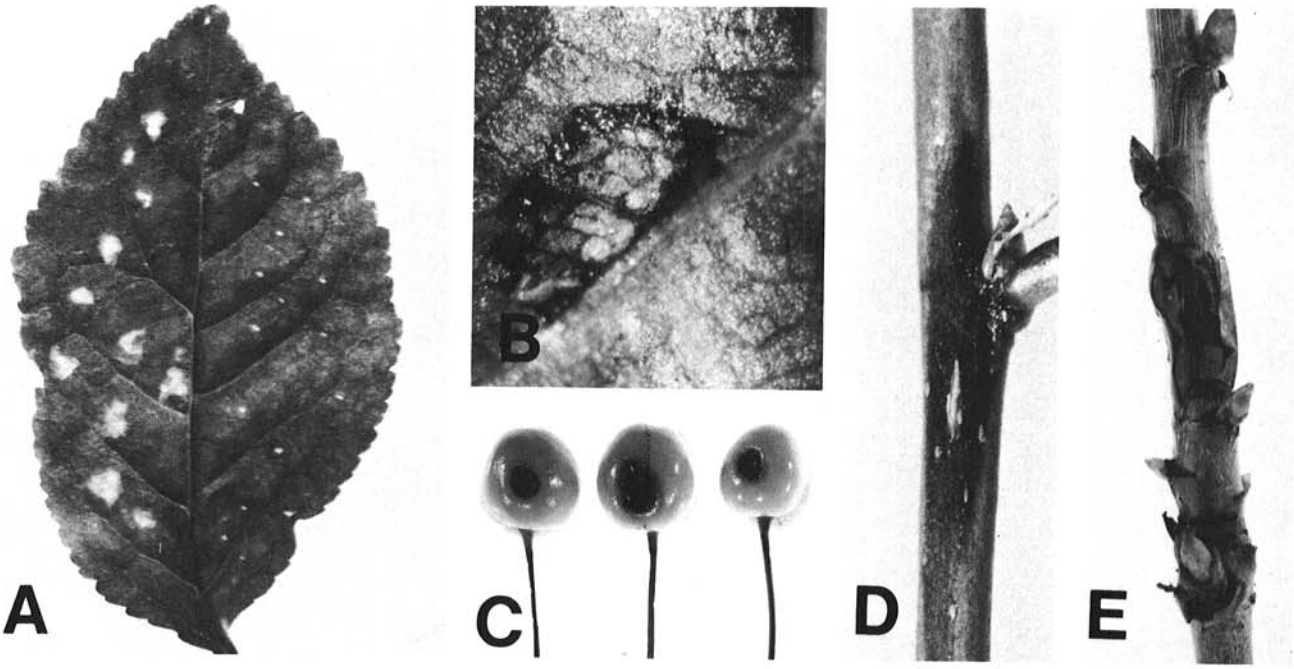

Fig. 2. Symptoms of bacterial canker obtained after artifical inoculations on Montmorency sour cherry (A, B, D, and E) and sweet cherry (C). **A**, Angular leaf spot (left) and necrotic specks obtained with sterile water (right). **B**, Water soaking 2–3 days after inoculation on the undersurface of the leaf. **C**, Dark brown and soft spots on the fruits. **D**, Dark brown necrotic spot around the inoculation site on green shoots. **E**, Cankers on woody stems.

TABLE 2. Additional physiologic properties of isolates from symptomless Montmorency sour cherry in Michigan

Group	Lactate	Arbutin	Growth on sucrose broth[a]			Syringomycin	Phage A7
			Yt	Yc	Wc		
GATTa$^+$[b]	157/165[c]	99/105	26/33	7/33	0/33	132/177	23/111
GATTa$^-$	1/130	3/57	0/36	0/36	36/36	3/127	94/100

[a] Determined in 50 ml of nutrient broth containing 5% (w/v) sucrose. Characteristics of the supernatant fluid were: Yt = yellow and translucent, Yc = yellow and cloudy, and Wc = white and cloudy.
[b] The GATTa$^+$ isolates are positive for gelatin liquefaction and aesculin hydrolysis but negative for tyrosinase activity and tartrate utilization. The GATTa$^-$ isolates are negative for gelatin liquefaction and aesculin hydrolysis but positive for tyrosinase activity and tartrate utilization.
[c] Number of positive isolates of the total number tested.

tissue, 34 were GATTa$^-$ and 2 were GATTa$^+$. *P. syringae* reference strains were characterized as GATTa$^+$, but *P. morsprunorum* strains were GATTa$^-$.

About 95% and 100% of the GATTa$^+$ isolates recovered from symptomless and diseased cherry samples, respectively, were positive for L (+) lactic acid utilization and β-glucosidase activity on arbutin agar medium; they yielded a yellow translucent or yellow and cloudy supernatant fluid in sucrose nutrient broth (Table 2). Conversely, about 99% of the GATTa$^-$ isolates from symptomless cherry samples and all the GATTa$^-$ isolates from diseased tissue did not utilize L (+) lactic acid as a sole carbon source. All GATTa$^-$ isolates from diseased samples were negative for β-glucosidase activity on arbutin medium, but 5.3% of the GATTa$^-$ isolates from symptomless samples were positive for β-glucosidase activity. All GATTa$^-$ isolates yielded a white, cloudy supernatant fluid in sucrose nutrient broth (Table 2).

Of 177 GATTa$^+$ isolates, 132 (74.5%) produced syringomycin on PDA. Most GATTa$^-$ isolates were negative for syringomycin activity (Table 2). The majority (79%) of the GATTa$^+$ isolates were not lysed by phage A7. However, 23 exceptions were found among the 111 GATTa$^+$ isolates. Conversely, most (94%) of the GATTa$^-$ isolates were lysed by phage A7 (Table 2). None of the reference strains of *P. syringae* was lysed by phage A7.

Most GATTa$^+$ and GATTa$^-$ isolates were positive for levan formation, developing dome-type colonies after 3–4 days. Twenty GATTa$^+$ and 20 GATTa$^-$ isolates utilized citrate, malic acid, D-sorbitol, and i-erythritol as a sole carbon source, but they did not utilize maleic acid. Acid production on purple lactose agar was erratic.

Pathogenicity. Of the 426 isolates recovered from symptomless sour cherry tissue, 302 induced a hypersensitive reaction on tobacco leaves in 24 hr (Table 3) and 230 of 321 were pathogenic to sweet cherry fruits, inducing dark brown and sunken lesions of variable size 3–4 days after inoculation (Fig. 2C). All isolates from diseased tissue samples induced a hypersensitive reaction on tobacco and were pathogenic to cherry fruits. Identical symptoms were obtained when sweet cherry fruits were inoculated at concentrations of 1×10^5 and 1×10^4 cells per milliliter. Fruits inoculated with nonpathogenic isolates only developed a light brown necrotic speck at the inoculation site. Of 241 isolates, 83 produced light brown lesions on bean pods, typical of incompatible *P. syringae* strains (9). Most lesions on bean fluoresced under ultraviolet light and some isolates also induced a brown reddish coloration around the inoculation site.

Isolates from diseased samples and symptomless buds, blossoms, leaves, and branches were pathogenic on sour cherry leaves, inducing water soaking lesions 3–4 days after inoculation (Fig. 2B). Necrotic lesions with chlorotic halos developed in 7–10 days (Fig. 2A). Occasionally, the necrotic tissue dropped out of the leaves and left shot holes. Of the 43 pathogenic isolates tested, 18 were GATTa$^+$ and 25 were GATTa$^-$. Of 40 isolates from buds, flowers, leaves, and branches, 31 were pathogenic on shoots of sour cherry, inducing dark brown necrotic lesions within 2 wk (Fig. 2D). Seven of the 31 isolates were GATTa$^+$ and 24 were GATTa$^-$. Four of eight isolates that infected sour cherry leaves were pathogenic on succulent sour cherry shoots at concentrations of 1×10^7 and 1×10^5 cells per milliliter; one isolate induced symptoms only at a concentration of 1×10^7 cells per milliliter, and three isolates did not induce symptoms. Five of seven isolates induced dark brown necrotic lesions on woody stems (Fig. 2E). Gummosis developed in 20–30 days.

Only 12 of 271 isolates (4.8%) from symptomless and diseased samples did not give a hypersensitive reaction on tobacco but were pathogenic to sweet cherry fruits (Table 4). Of 12 isolates, five were GATTa$^+$ and seven were GATTa$^-$. Conversely, 20 isolates (7.4%) that induced hypersensitivity on tobacco failed to infect sweet cherry fruits (Table 4). Four isolates were GATTa$^+$ and 16 were GATTa$^-$. Finally, 14 of 271 isolates (5.2%) failed to induce hypersensitivity or to infect sweet cherry fruits.

Of the 76 GATTa$^\pm$ isolates (Table 1), 16 induced a hypersensitive reaction on tobacco and nine infected sweet cherry fruits.

Of 177 GATTa$^+$ isolates from symptomless samples, 112 (63.3%) induced hypersensitivity on tobacco and produced syringomycin. Thirteen isolates (7.3%) were negative for both hypersensitivity on tobacco and syringomycin production. Twenty-eight isolates (15.8%) induced a hypersensitive reaction on tobacco but failed to produce syringomycin; of the latter isolates 19 infected sweet cherry fruits.

Ice-nucleation. Ice-nucleation activity was positive for 51 of 105 (48.6%) GATTa$^+$ isolates and 23 of 96 (24%) GATTa$^-$ isolates.

TABLE 3. Pathogenicity of Gram-negative, oxidase-negative, and fluorescent bacteria recovered in 1977-1978 from symptomless Montmorency sour cherry collected in two orchards in southwest Michigan and in one orchard in west central Michigan

Source of isolates	Tobacco hypersensitivity		Pathogenicity to cherry fruit		Green bean pod reaction	
	Ratio[a]	(%)	Ratio	(%)	Ratio	(%)
Buds	43/60	72	28/30	93	18/20	90
Flowers	39/50	78	31/43	72	3/20	15
Fruits	24/36	67	25/36	69	8/26	31
Leaves	113/150	75	85/111	77	24/105	23
Branches	83/130	64	61/101	60	30/70	43
Totals	302/426		230/321		83/241	
%		71		72		34

[a]Number of isolates giving a positive reaction over total number tested.

DISCUSSION

The GATTa tests were useful for rapid identification of *P. syringae* and *P. morsprunorum*. The four physiologic tests are practical and, with the exception of tyrosinase determination, provided objective and consistent results. The tyrosinase test sometimes was difficult to interpret because green fluorescent pigment also is produced, making it difficult to distinguish the brown reddish pigmentation indicative of tyrosinase activity. Based on the results of several physiologic tests (Table 2), the GATTa$^+$ and GATTa$^-$ isolates conformed to the characteristics described for *P. syringae* and *P. morsprunorum*, respectively (1,2,5,6,10,14,23).

TABLE 4. Relationship between tobacco hypersensitivity test and pathogenicity of 251 isolates from symptomless Montmorency sour cherry and 20 isolates from diseased cherry trees[a] on immature sweet cherry fruit

Tobacco hypersensitivity	Pathogenic to sweet cherry fruits							
	Positive				Negative			
	GATTa$^+$[b]	GATTa$^-$[b]	Total	(%)[c]	GATTa$^+$	GATTa$^-$	Total	(%)
Positive	119	106	225	83.0	4	16	20	7.4
Negative	5	7	12	4.4	11	3	14	5.2

[a]Disease samples were obtained from Montmorency sour cherry and Vega and Napoleon sweet cherry trees.
[b]GATTa$^+$ isolates are positive for gelatin liquefaction and aesculin hydrolysis but negative for tyrosinase activity and tartrate utilization. GATTa$^-$ isolates are negative for gelatin liquefaction and aesculin hydrolysis but positive for tyrosinase activity and tartrate utilization.
[c]Percent of 271 isolates tested.

The GATTa$^{\pm}$ isolates comprise a heterogenous group and the significance to bacterial canker of sour cherry remains to be established. They exhibit some properties of both *P. syringae* and *P. morsprunorum* and illustrate the difficulty of using a set of determinative tests to identify *P. syringae* and *P. morsprunorum* and the need to test a relatively large number of isolates from the population.

Syringomycin activity appears to be fairly specific for GATTa^{+} isolates and was rarely found in GATTa^{-} or GATTa^{+} isolates. Although syringomycin activity and the tobacco hypersensitivity reaction have been suggested as indicative of the pathogenicity of *P. syringae* (11,16), our data indicate a potential error in using these tests alone to determine pathogenicity (Tables 2 and 4).

P. morsprunorum, but rarely *P. syringae*, was isolated from diseased sour cherry tissue collected in 1976 and 1977, which indicates that the former species was responsible for the bacterial canker epidemic of sour cherry during the two seasons. Nevertheless, *P. syringae* should not be disregarded because pathogenic isolates were recovered throughout the growing season from symptomless sour cherry samples and because outbreaks of *P. syringae* were noted previously on sweet cherry in Michigan (14).

Pathogenic *P. morsprunorum* and *P. syringae* isolates, in about a 1:1.4 ratio, were consistently recovered from symptomless sour cherry buds, flowers, fruits, leaves, and branches throughout the season and long before symptoms appeared, demonstrating that both organisms are well distributed epiphytically (sensu Leben [18]) on host tissue, confirming previous studies (3,4,8,9). Cankers (24) and resident populations (5,8,9,22) have been proposed as sources of inoculum on sweet cherry and other fruit crops. Because cankers are seldom found in sour cherry orchards, resident populations should be regarded as an important source of inoculum for bacterial canker because of their broad distribution and rather consistent presence throughout the season.

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