New Medium for Detecting *Erwinia amylovora* and Its Use in Epidemiological Studies

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ABSTRACT

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A medium, CCT, was developed that distinguishes *Erwinia amylovora* from *E. herbicola* on the basis of colony morphology. Accuracy is such that one colony-forming unit (cfu) of *E. amylovora* per milliliter can be detected in the presence of 10^4 cfu of *E. herbicola* per milliliter on CCT. Forty-five of 48 virulent strains of *E. amylovora* tested had similar colony morphology on CCT, while numerous other bacterial species have dissimilar morphologies. CCT was successfully used to detect *E. amylovora* in apple blossoms, buds, and cankers prior to development of fire blight symptoms.

Additional key words: *Malus malus*.

Selection and differentiation of *Erwinia amylovora* (Burrill) Winslow et al., the causal organism of fire blight on rosaceous plants, has been demonstrated on various media (2,4,6,8). Monitoring *E. amylovora* prior and subsequent to symptom development in apple and pear orchards has been useful in some areas for forecasting fire blight epiphytotics (1,4). In Michigan, similar studies were not successful due to high populations of *E. herbicola* (Lohnis) Dye, a saprophytic bacterium, in the sampled tissue. When the number of colony-forming units (cfu) of *E. herbicola* on the media used to monitor *E. amylovora* was greater than the number of cfu of *E. amylovora*, accurate detection of *E. amylovora* was greatly reduced (7).

This paper reports the development of a medium (CCT) to detect and enumerate *E. amylovora* in the presence of *E. herbicola* and other bacteria that are commonly isolated from apple tissue.

MATERIALS AND METHODS

Media. CCT medium was made by combining 100 g of sucrose, 10 g of sorbitol; 30 ml of a 1% aqueous solution of tergitol anionic 7, 2 ml of a 0.1% solution of crystal violet in absolute ethanol, 23 g of nutrient agar (Difco), and 970 ml of distilled water. Two milliliters of a 1% (w/v) thallium nitrate solution and 50 mg of cycloheximide were added after the medium was autoclaved and cooled to 50 C. Plastic petri dishes were then poured with 20 ml of CCT and could be stored in the dark at 5 C for 2–3 wk. Longer storage is not recommended because CCT can become toxic to *E. amylovora* upon prolonged storage.

Other media used for comparison were: nutrient agar (Difco) with 0.5% glucose (NGA), Crosse and Goodman medium (C+G) (2), 5% sucrose agar (8), TTN (6), and nutrient broth (Difco) with 0.5% glucose (NGB). *Erwinia herbicola* was maintained on YCA medium which is comprised of 10 g of yeast extract (Difco), 2.5 g of calcium carbonate, and 15 g of Bacto agar per liter. Yellow bacteria were isolated from plant parts on YCA. All inoculated media were incubated at the recommended temperatures and length of time. CCT plates were incubated at 27 C for 3 days. YCA plates were incubated for 2 days at 37 C. In all cases, 20 mM potassium phosphate buffer, pH 6.8, was used for isolations and dilutions.

Bacterial isolates. Isolates of *E. amylovora* were obtained from culture collections around the world (Table 1). Other bacteria were obtained from cultures maintained in the Department of Botany and Plant Pathology at Michigan State University.

Morphology of bacterial colonies on CCT. A suspension of each isolate was made by placing a loopful of bacteria from a 24–48-hr culture on NGA into phosphate buffer. Ten-fold serial dilutions in buffer were made and 0.1 ml of the appropriate dilutions was spread with a sterile bent glass rod on CCT and NGA plates. Plates were incubated at 27 C and results were recorded at 2, 3, and 4 days. Each isolate was plated on CCT at least twice.

Plating efficiency. *E. amylovora* isolates 110 Rif^r, Ea9, Ea1, and Ea101b were grown in 25 ml NGB for 18 hr and serially diluted in phosphate buffer. One-tenth milliliter of each dilution was spread onto plates of TTN, CCT, C+G, 5% sucrose, and NGA. Three plates of each dilution were plated. This experiment was repeated three times.

Enumeration of *E. amylovora* in mixed cultures. NGB cultures of *E. herbicola* 13 and *E. amylovora* 110 Rif^r were grown to yield 3×10^8 cfu/ml. The two broths were adjusted in phosphate buffer and mixed to yield final concentration ratios of approximately 1,000:1, 100:1, 10:1, and 1:1 *E. herbicola* to *E. amylovora*, respectively. These bacterial suspensions were serially diluted in phosphate buffer and plated on NGA, TTN, C+G, 5% sucrose agar, and CCT. Each dilution was plated twice. Results were recorded at 2, 3, and 4 days. C+G medium was viewed under a dissecting scope for enumeration of *E. amylovora*. This experiment was repeated three times.

Field isolations. Three apple orchards were included in the 3-yr study. Orchards 1 and 2, both apple *Malus malus* 'Jonathan' were located on the Michigan State University Botany farm, East Lansing, MI, and were 23 and 15 yr old, respectively. Orchard 3, of cultivar Ida Red, was located in Leslie, MI, and was 5 yr old. No bactericides were used in the orchards and all had a history of fire blight.

Orchard 1 was sampled in 1979 at 80% bloom and at petal fall for *E. amylovora* and yellow bacteria. Samples consisted of 75 flowers collected randomly from three trees. Five samples were obtained from five sets of three trees.

Orchard 2 was sampled three times in the spring of 1980. The first sample was collected at the green tip stage; 27 samples of 10 buds each were collected from nine trees, three samples per tree. At 50 and 80% bloom, 15 samples containing 25 fully expanded flowers were collected from 15 trees. The fifteen trees were used for blight assessment and as a source of cankers in 1981.

Cankers tagged at Orchard 2 in 1980, were removed on 23 March and 8 April, 1981. They resulted from terminal blight in 1980 on twigs 0.5–1.0 cm in diameter and had definite margins. At each sample date, 32 cankers were assayed for *E. amylovora*.

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Orchard 3 was sampled in 1981 for *E. amylovora* in flowers at four different times beginning at the pink stage and continuing through petal fall. Blossoms were collected from 10 trees, 15 flowers per tree.

Green tip buds and flower samples for isolation were placed in a plastic bag and held on ice until they could be processed. Tissues were cut into 0.5-cm pieces and soaked in phosphate buffer for 1 hr on a shaker, 80 strokes per minute. Approximately 1.5 ml of buffer per flower or bud was added to the sample. Cankers with 0.5 cm on each side of the canker margin were aseptically removed, cut into 0.2-mm pieces, and soaked in 10 ml of phosphate buffer for 1 hr.

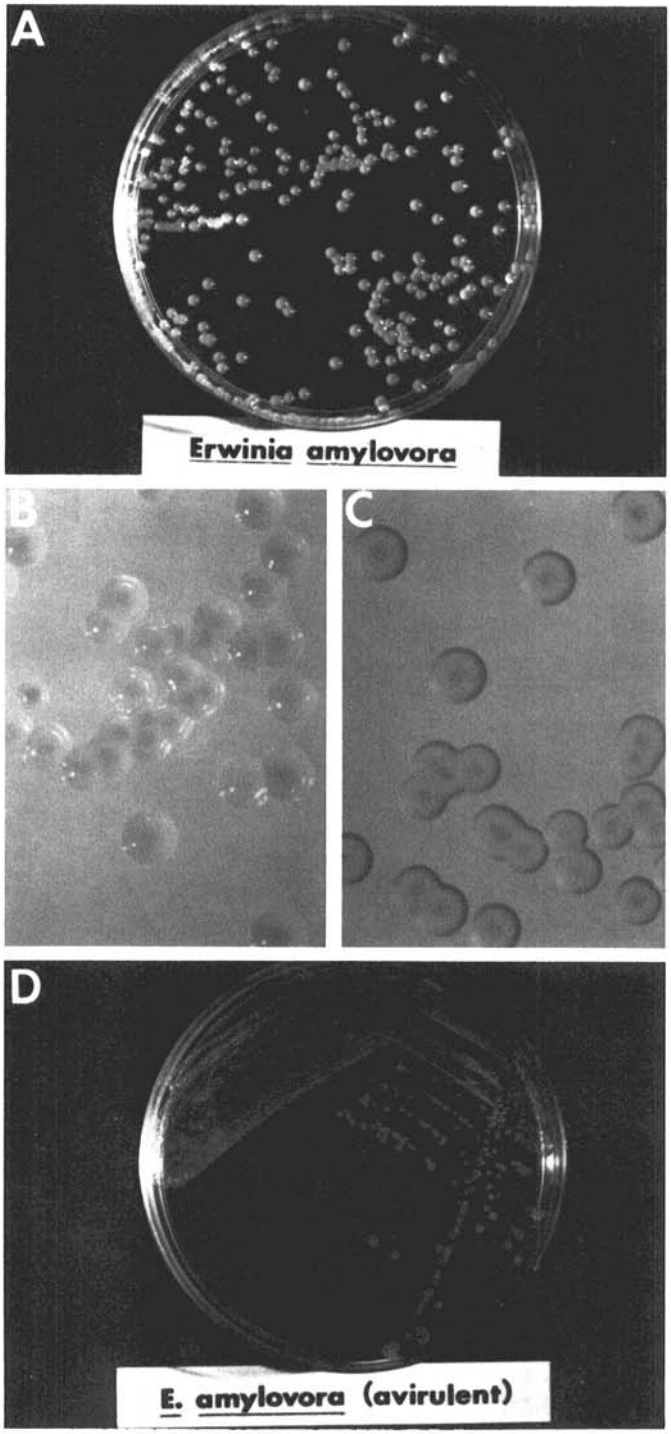

Fig. 1. Colony morphology of *Erwinia amylovora* on medium CCT. **A,** Typical colony morphology of a virulent isolate of *E. amylovora* on CCT. **B,** Magnified top view of **A**. **C,** Magnified view of the underside of the colonies, showing a faint cluster of rays in the center of each one. **D,** Avirulent colony type.

Samples were diluted in phosphate buffer and 0.1 ml of three different dilutions was spread with a bent glass rod on CCT plates for the isolation of *E. amylovora* and on YCA plates incubated at 37 C for yellow bacteria. Each dilution was plated twice.

Identification of *E. amylovora* on CCT. After incubation on CCT for 3 days, plates were placed under a long-wave UV light source to observe any fluorescing colonies. Colonies that appeared pulvinate, light blue, and nonfluorescent and that had blue striations radiating from the center of the colony were considered to be *E. amylovora* (Fig. 1). A random selection of isolates thought to be *E. amylovora* on CCT was further identified by Gram stain, facultative anaerobic growth in thioglycolate medium, and pathogenicity on Jonathan apple seedlings (5).

RESULTS

Colony morphology of bacterial isolates on CCT. Forty-eight virulent isolates of *E. amylovora* obtained from culture collections were tested on CCT. All of these except three had similar colony morphology (Fig. 1). After 3 days, smooth, large (4.0–7.0-mm), pulvinate, light blue opalescent colonies with entire margins were evident. When viewed from the underside of the petri dish, blue striations radiating from the center of the colonies could be seen. After 4 days on CCT, some isolates collapsed and individual colonies were difficult to see. All three isolates that did not have the typical morphology of *E. amylovora* were from pear and were unlike each other in appearance on CCT. Three avirulent isolates of *E. amylovora* were tested on CCT. Three-day-old avirulent colonies were blue, slightly raised, and 3.0–5.0 mm in diameter with contoured surfaces, contorted blue rays, and irregular margins.

On CCT, other bacterial isolates exhibited different colony morphology than *E. amylovora*. The nine isolates of *E. herbicola* that were tested differed markedly from *E. amylovora* on CCT. After 2 days, colonies of *E. herbicola* were yellow with a blue margin or entirely blue, 3.0–4.0 mm in diameter, slightly raised, and with entire to erose margins and contoured surfaces. *Corynebacterium fasciens, C. flaccumfaciens, Xanthomonas campestris, X. pelargonii, X. phaseoli* 11, *X. phaseoli* 24, and *X.*

TABLE 1. Sources of *Erwinia* spp. isolates tested on medium CCT

Source	*Erwinia* spp.	Culture collection no.
R. N. Goodman	*E. amylovora*	Ea8, Ea9, 101b
R. Covey	*E. amylovora*	Ea1415, Ea1286, Ea1565
M. N. Schroth	*E. amylovora* *E. herbicola*	Ea812 UCBPP842, UCBPP843
D. Ritchie	*E. amylovora*	B6
D. Berry	*E. amylovora*	6/80
S. V. Beer	*E. herbicola* *E. amylovora*	Eh103, Eh125, Eh145 Ea261, Ea246, Ea271, Ea308, Ea292, Ea235, Ea273, Ea263, Ea109, Ea307
S. Ries	*E. amylovora*	Ea4, Ea5, EaPC457
J. P. Paulin	*E. amylovora*	Ea1377, Ea1430, Ea1365, Ea1368, Ea1431
D. W. Dye	*E. amylovora*	PDDCC550, PDDCC1494, PDDCC1841, PDDCC1530, PDDCC1534, PDDCC1540, PDDCC3859
E. Billings	*E. amylovora*	EaT, EaAT, EaP70, Ea3A2B
W. Zeller	*E. amylovora*	Ea4/79, Ea1/79, Ea6/79, Ea1/80
G. Dinesen	*E. amylovora*	Ea671, Ea638, Ea623
H. P. Maas Geesteranus	*E. amylovora*	Ea117, Ea122, Ea209, Ea211, Ea214
D. Coplin	*E. stewartii*	SS104, SW2

pruni did not grow on CCT. *Agrobacterium tumefaciens, Bacillus megaterium, Escherichia coli, Erwinia carotovora*, and *E. stewartii* grew on CCT. Colonies from these species were all <3.0 mm in diameter and did not exhibit the pulvinate character of *E. amylovora*. Eighteen pseudomonads were tested. None of the nonfluorescing species grew on CCT. Of the fluorescing group, seven grew on CCT. These were either *P. syringae* or *P. fluorescens*. Only three of these seven isolates were similar to *E. amylovora*. These isolates were pulvinate and light blue after 2 days on CCT. Pseudomonads could be distinguished from *E. amylovora* by the complete lack of striations or the appearance of blue concentric rings in the center of the colony, faster growth than *E. amylovora*, and fluorescence of colonies after 2–4 days when viewed under a long-wave UV light source in the dark.

Plating efficiency. There were no significant differences ($P = 0.05$) in the number of cfu of *E. amylovora* obtained on any of the media tested.

Enumeration of *E. amylovora* in mixed cultures. Significant differences ($P = 0.01$) were found between the media in recovery of *E. amylovora* in the presence of *E. herbicola*. Orthogonal contrast analysis indicated that the slopes of the growth curves on TTN, NGA, C+G, and 5% sucrose agar were significantly quadratic (Fig. 2). CCT had significant linearity and no quadratic component in its slope. These results suggest CCT detected *E. amylovora* efficiently over the range of ratios of *E. herbicola* to *E. amylovora* tested. The other media tested recovered *E. amylovora* efficiently only when the cfu ratio of *E. herbicola* to *E. amylovora* did not exceed 10^2. CCT recovered *E. amylovora* efficiently when this ratio did not exceed 10^4.

Orchard isolations and symptom development. *E. amylovora* was recovered on CCT from flower samples taken from Orchard 1 prior to symptom development in 1979 (Table 2). Three of five samples collected at 80% bloom on May 16 harbored *E. amylovora* at dilution concentrations ranging from 10 to 10^2 cfu/ml. Samples collected on 23 May at petal fall detected *E. amylovora* in five of five samples at 10^5 to 10^6 cfu/ml. Other bacteria were present at both sample dates. A yellow bacterium that grows at 37 C was the most frequently observed. Yellow bacteria were recovered in eight of the 10 samples and in greater number than *E. amylovora*. Blossom blight developed in Orchard 1 on 2 June. An average of five blighted blossom spurs per tree was counted on the 15 trees sampled.

E. amylovora was also detected on CCT before symptom development at Orchard 2 in 1980 (Table 2). *E. amylovora* was detected in only one sample of green tip buds at the first dilution corresponding to 10 cfu/ml. Yellow bacteria were present in 12 green tip bud samples at dilution concentrations of 10 to 10^3 cfu/ml. At 50% bloom on 19 May, *E. amylovora* was detected in six of 15 samples. Levels of *E. amylovora* ranged from 10 to 10^2 cfu/ml. Yellow bacteria were present in all 15 samples at 10 to 10^4 cfu per flower. No blight was apparent at this sampling date. *E. amylovora* was found in nine of 15 samples collected at 80% bloom on 22 May. The pathogen was detected in the 10 to 10^4 cfu/ml dilutions. Yellow bacteria were present in all samples at the 10^2 to 10^6 cfu/ml dilutions.

The first visible blight symptoms in Orchard 2 were seen on 1 June 1980. One fruit pedicel had small droplets of white ooze along its length. By 7 June, numerous vegetative terminals and fruit spurs were blighted. Orchard 2 had a high incidence of blight in 1980. Blight assessments for 15 trees at terminal bud set revealed an average of 33/50 tagged terminals were blighted.

E. amylovora was detected in 21 of the 64 cankers sampled from Orchard 2 in the 10 and 10^2 cfu/ml dilutions (Table 2). Yellow bacteria were not assayed.

A 1981 flower survey in Orchard 3 did not detect *E. amylovora*. Blossom blight did not develop in this orchard, and the incidence of terminal blight was very low. Yellow bacteria were not assayed.

DISCUSSION

We have developed a differential medium, CCT, for the detection and enumeration of *E. amylovora* in the presence of *E. herbicola*. With few exceptions, isolates of *E. amylovora* exhibited a typical colony morphology distinguishable from other bacterial species. Some isolates of *P. fluorescens* and *P. syringae* are pulvinate and light blue on CCT, but these can be distinguished from *E. amylovora* by fluorescence under a long-wave UV light source, rapid growth, and lack of striations or the presence of concentric blue rings. A similarity between colony morphology of

TABLE 2. Detection on CCT of *Erwinia amylovora* in the presence of yellow bacteria in samples from Michigan apple orchards

Sample description	Samples (no.)	Samples of *E. amylovora* detected (no.)[a]	Dilution range of *E. amylovora* (cfu/ml)[b]	Samples of yellow bacterium detected (no.)	Dilution range of yellow bacteria (cfu/ml)
Orchard I					
16 May 1979 80% bloom, 75 flowers per sample	5	3	$10–10^2$	5	$10^2–10^4$
23 May 1979 petal fall, 75 flowers per sample	5	5	$10^5–10^6$	3	$10^4–10^5$
Orchard II					
23 March 1980 green tip buds, 10 buds per sample	27	1	10	12	$10–10^3$
19 May 1980 50% bloom, 25 flowers per sample	15	6	$10–10^2$	15	$10–10^4$
22 May 1980 80% bloom, 25 flowers per sample	15	9	$10–10^4$	15	$10^2–10^5$
23 March 1981 cankers	32	9	$10–10^2$	nd[c]	
8 April 1981 cankers	32	12	$10–10^3$	nd	

[a] Each sample was diluted in phosphate buffer and 0.1 ml was plated on CCT and YCA. CCT plates were incubated at 27 C for 3 days. YCA plates were incubated at 37 C for 2 days. *E. amylovora* was enumerated on CCT. The yellow bacterium was enumerated on YCA.
[b] Numbers represent the highest and lowest dilutions in which *E. amylovora* or the yellow bacterium was detected.
[c] nd = not done.

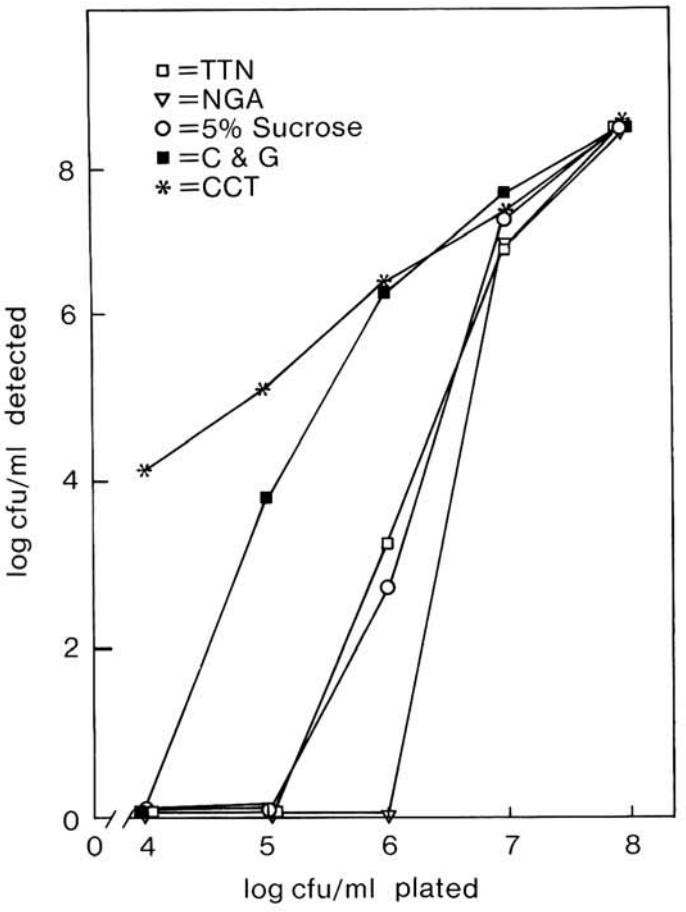

Fig. 2. Log colony-forming units (cfu) of *E. amylovora* detected per milliliter (ordinate) in mixed culture suspensions containing 3×10^8 cfu of *E. herbicola* per milliliter and 10-fold dilutions of *E. amylovora* ranging from 3×10^4 to 3×10^8 cfu of *E. amylovora* per milliliter (absissa) plated on five different media.

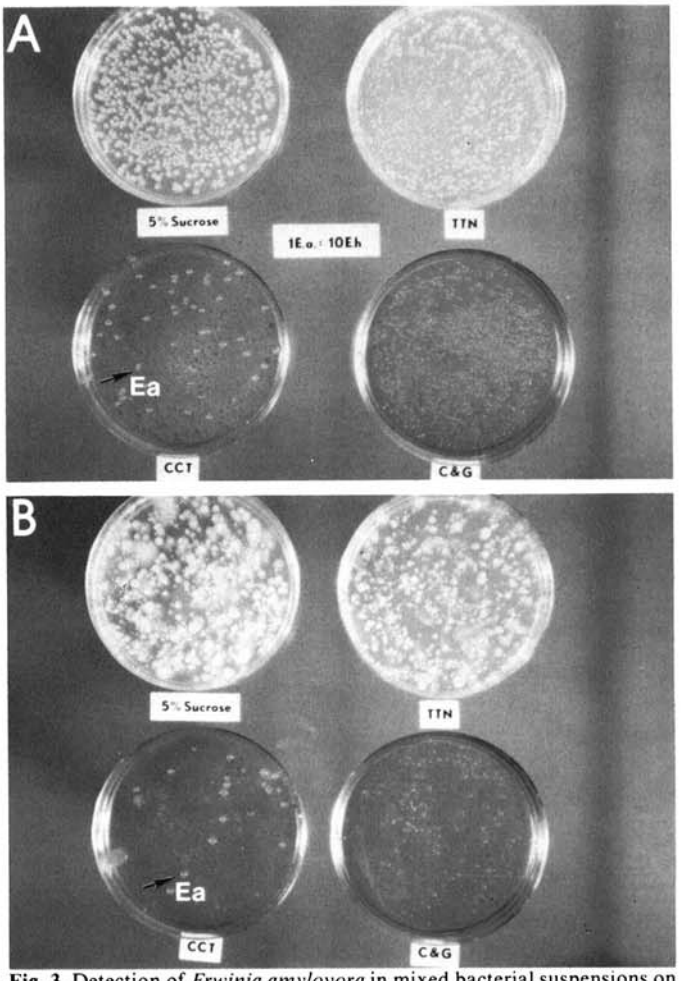

Fig. 3. Detection of *Erwinia amylovora* in mixed bacterial suspensions on four different media. **A,** A suspension of *E. amylovora* and *E. herbicola* in the ratio 1:10 cfu/ml plated on each medium. The large white colonies (arrow) are *E. amylovora* and the flat, dark colonies are *E. herbicola* on medium CCT. **B,** Isolation from blighted apple tissue soaked for 1 hr in buffer of which 0.1 ml was plated on each medium. The large white pulvinate colonies (arrow) on CCT are *E. amylovora*.

E. amylovora and *P. syringae* has also been noted on 5% sucrose medium and is due to levan production (8).

In vitro experiments demonstrated the efficiency of CCT in enumeration of *E. amylovora* in the presence of *E. herbicola*. Recovery of *E. amylovora* was greater on CCT than on NGA, TTN, C+G, or 5% sucrose media. MS agar (4) was not used in the experiment because of difficulties reported by other investigators (7) and our difficulty in distinguishing *E. herbicola* from *E. amylovora* in mixed cultures on MS agar.

In contrast to earlier reports (7), *E. amylovora* was recovered on CCT from dormant apple buds, flowers, and terminals in Michigan before symptoms appeared in the orchards. Yellow bacteria able to grow at 37 C were routinely isolated from these samples and were usually isolated at higher concentrations from the tissues than *E. amylovora*. *E. amylovora* was also recovered from small 1-yr-old cankers. These results support the hypothesis that cankers <1 cm wide contribute to inoculum potential in the spring (1,7). Ability to detect *E. amylovora* in the presence of a greater number of yellow, saprophytic bacteria before the appearance of symptoms may be useful in the forecasting of fire blight epidemics.

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