A New Selective Medium for *Pseudomonas solanacearum*

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ABSTRACT

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A medium (SM-1) with good plating efficiency and high selectivity was developed and used to isolate *Pseudomonas solanacearum* from artificially and naturally infested soils. The medium was prepared by adding to Kelman's tetrazolium chloride (TZC) medium the following: crystal violet (50 μg/ml), thimerosal (5 μg/ml), polymyxin B sulfate (100 μg/ml), tyrothrycin (20 μg/ml), and chloromycetin (5 μg/ml). Most fungi did not grow on this medium. With some soil samples, however, adding either cycloheximide (50 μg/ml) or chlorothalonil (80 μg/ml) to the medium eliminated the problem of fungal contamination. After 2–3 days of growth at 30 C, colonies of *P. solanacearum* on this medium were round, pulvinate, fluidal, and milky white. For 19 of 20 strains tested, plating efficiency of SM-1 ranged from 81 to 138% of that of TZC. These strains included representatives of the three races of the pathogen; only one strain of race 3 (119, from Costa Rica) grew poorly on SM-1 (41% plating efficiency). Growth of most soil bacteria was inhibited (more than 99% in 11 soil samples containing 1.02×10^7–1.03×10^8 cfu/g dry wt) on SM-1. In one soil sample from Costa Rica, however, only 67.4% of the bacterial population was inhibited. The good plating efficiency and marked reduction of background bacteria obtained with SM-1 allowed recovery of *P. solanacearum* from soils containing as few as 2.2×10^2 cfu/g. The bacterium was isolated easily from naturally infested soils obtained from various locations in the United States and Costa Rica.

Bacterial wilt caused by *Pseudomonas solanacearum* E. F. Smith is one of the most destructive bacterial diseases of plants in the tropics and subtropics. Early work on control of *P. solanacearum* by crop rotation led to the concept that the bacterium can survive in soil for several years (19). When survival has been measured directly, however, it has been shown that the bacterium does not remain in soil for periods longer than a few weeks (5).

The bacterium has been detected in soil either indirectly or directly. With the indirect methods, incidence of the disease on susceptible hosts planted at various intervals is determined (7,11,17,21) or tobacco leaves are infiltrated with soil extracts (21). With the direct methods, the bacterium is isolated from soil by means of selective media (3,5,9,16) or is detected by serological techniques (8). Use of selective media has been the most effective means to detect the bacterium in the soil, but none of the selective media developed so far have gained wide acceptance because they 1) allow growth of too many background bacteria, as in the case of Okabe's (15), Okabe's modified (1), and Drigalski's modified (22) media; 2) are appropriate only for certain strains of *P. solanacearum*, as in the case of the Harris' (7) and Karganilla and Buddenhagen's (9) media; or 3) are difficult to prepare, as in the case of the medium of Nesmith and Jenkins (14).

Lack of an efficient selective medium that can be used with different types of soils is one of the reasons most studies on survival of *P. solanacearum* have been carried out by indirect methods for detecting the bacterium (2,13,18). Also, this may be the reason very little is known about the behavior of the bacterium in the rhizosphere of host and nonhost plants.

Our initial attempts to use several selective media developed by others (7,9,14) for *P. solanacearum* were not successful for isolation of the bacterium when present at low concentrations in rhizosphere soil. It was decided, therefore, to develop a new selective medium that combined some of the features of the media that were available but with greater selective capacity.

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MATERIALS AND METHODS

Cultures. All strains of *P. solanacearum* used in this work (Table 1) were from the culture collection maintained in the Department of Plant Pathology, University of Wisconsin, Madison. Stock cultures were maintained in sterile distilled water at room temperature. To prepare fresh inoculum, stock suspensions were streaked on Kelman's tetrazolium chloride agar medium (TZC) (10) and incubated at 30 C for 48 hr.

Development of a selective medium. In preliminary tests, several antimicrobial compounds, including antibiotics (bacitracin, chloromycetin, polymyxin B sulfate, novobiocin, tyrothricin, and vancomycin, all from Sigma Chemical Co., St. Louis, MO 63178), fungicides (benomyl, chloroneb, chlorothalonil, cycloheximide, pentachloronitrobenzene, and dichloran), and bactericides (thimerosal, rose bengal, and brilliant green, all from Diamond Products, Inc., Seffner, FL) were assayed for effectiveness as selective agents when added at different concentrations and combinations to TZC medium. Antibiotics and fungicides were sterilized by dissolving in 70% ethanol (1 ml) and held for 30 min (14), then diluted to the desired concentration with sterile distilled water and stored at 4 C. Tetrazolium chloride and crystal violet were autoclaved for 7 min at 121 C and 1.05 kg force per square centimeter. Thimerosal (merthiolate) was added without prior treatment. Each antimicrobial compound tested was added just before dispensing the medium into petri dishes.

Unidentified rhizosphere bacteria from pepper, castor bean, corn, and bean were streaked individually on plates of each medium being assayed and those able to grow were tested further against a set of chemotherapeutants (Bacto-sensitivity disks, Difco Laboratories, Detroit, MI) at low and high concentrations that included the following: pimaricin, erythromycin, terramycin, ampicillin, elkosin, furadantin, viomycin, kanamycin, sulfathiazole, oxacillin, tobramycin, gantricin, aureomycin, neomycin, rifampin, cephalothin, doxicycline, nalidixic acid, and oxilinic acid. Disks were placed on plates of CPG medium (casamino acids 1 g/L, peptone 10 g/L, and glucose 5 g/L) containing rhizosphere bacteria (1×10^8 cfu/ml). After 48 hr of incubation at 30 C, the diameter (mm) of the zone of inhibition around each disk was used as a relative measure of sensitivity of rhizosphere bacteria to these compounds.

Plating efficiency. The effect of different combinations of antimicrobial compounds on growth of different strains of *P. solanacearum* was determined in solid media. Bacterial suspensions ($OD_{600\ nm} = 0.01$, diluted 10^{-4} and 10^{-5}) from 48-hr cultures of each strain were spread with a glass rod on three plates each of TZC medium and of the medium containing the antimicrobial compounds. To calculate plating efficiency, the average number of colonies on TZC medium at the same dilution was considered 100%. Combinations of

antimicrobial compounds that reduced plating efficiency of *P. solanacearum* were eliminated from the formula of the selective medium, and when possible, the compounds were tested again at lower concentrations. Media containing combinations of antimicrobial compounds that gave plating efficiencies equal to or greater than that of TZC medium were tested further for their effects on soil bacteria.

Percent reduction of soil bacteria. Soil samples from different geographical locations and from areas planted to different crops were used to determine the effectiveness of the selective medium in reducing the numbers of background bacteria. Ten-gram samples were placed in a volumetric flask and distilled water was added to the 100-ml mark and shaken for 20–30 min, then aliquots (0.1 ml) from 10-fold dilutions were spread on the medium to be tested. Dilutions were also spread on CPG medium to determine the total number of bacteria in the soil.

Percent reduction was calculated by dividing the number of bacteria recovered on the test medium by the total number of soil bacteria per gram of soil (dry weight) on CPG times 100, then subtracting from 100% (4). To determine the dry weight, 10 g of soil was dried to constant weight at 100–110 C. The medium with the highest plating efficiency and the lowest number of contaminating soil bacteria (see Results) was selected for further tests designed to detect *P. solanacearum* in the soil.

Recovery of *P. solanacearum* from artificially infested soil. To determine the efficiency of the selective medium in recovering *P. solanacearum* from soil, 1 ml of a bacterial suspension containing 10^2–10^5 cfu/ml was added to 1 g of soil, and 5–10 min later, 0.1-ml samples of 10-fold serial dilutions (usually 10^{-1} and 10^{-2}) were spread on each plate of the medium being evaluated. The actual number of *P. solanacearum* cells in the inoculum was determined by standard dilution plating on TZC medium. The total number of bacteria in the soil sample was also determined as described. This was done to relate the number of background bacteria to any potential problems in recovery of *P. solanacearum* from any given sample. Percent recovery was determined by dividing the number of bacteria recovered on the selective medium by the total number applied times 100.

Detection of *P. solanacearum* in naturally infested soil. Naturally infested soils obtained from Florida (Quincy), North Carolina (Clinton), Georgia (Athens), and Costa Rica (Turrialba) were used to determine the populations of *P. solanacearum* per gram dry weight of soil. Soil samples were provided by F. M. Shokes, University of Florida, S. F. Jenkins, Jr., North Carolina State University, S. M. McCarter, University of Georgia, and L. C. Gonzalez, University of Costa Rica. Procedures for isolating the bacterium were similar to those described for artificially infested soil.

RESULTS

The most selective combination of antimicrobial compounds and nutrients was designated SM-1. This medium was prepared by adding to TZC medium (at the time plates were poured) the following antimicrobial compounds: crystal violet (50 μg/ml), thimerosal (5 μg/ml), polymyxin B sulfate (100 μg/ml), tyrothricin (20 μg/ml), and chloromycetin (5 μg/ml). In the few instances where fungal growth occurred, adding either cycloheximide (50 μg/ml) or chlorothalonil (80 μg/ml) effectively eliminated this problem. Addition of antifungal compounds did not affect the effectiveness of the medium when compared with that without these compounds. Stocks of sterile antimicrobial compounds were stored at low temperature for not more than 90 days. The selective medium was stored for about 1 mo at 4 C without noticeable reduction in efficiency.

Colony appearance of *P. solanacearum* in the selective medium. Two to three days after plating on SM-1, colonies of *P. solanacearum* were milky white, round to oval, pulvinate to convex, and for the most part, entire and fluidal. By 4–5 days, the colonies had turned pink to reddish, depending on how much reduction of tetrazolium salt had occurred. At this time, colony morphology of the bacterium in SM-1 was similar to that on TZC medium. When some bacterial contaminants were present, colonies of *P. solanacearum* remained small, flat, and round or oval and the color changed to lavender or purple. These colonies appeared normal when replated on TZC medium.

Plating efficiency. When 20 strains of races 1, 2, and 3 were tested, the plating efficiency of SM-1 was equal to or even greater than that of TZC medium (Table 1). Because certain strains of both races 2 and 3 have some susceptibility to chloromycetin, the plating efficiency in those instances was reduced slightly (strains 98, 290, and 260) or drastically (strain 119). When dealing with these strains, therefore, the concentration of chloromycetin had to be adjusted according to their susceptibility.

Table 1. Plating efficiency of the selective (SM-1) medium for stock cultures of strains of *Pseudomonas solanacearum* from various hosts and geographical locations

Strain	Race	Location	Host	Donor	Plating efficiency[z] (%)
25	1	Wake County, NC	Tomato	A. Kelman, K60	111.0 b
86	1	Climax, GA	Tomato	R. E. C. Layne, 12A	102.1 bc
278	1	Mexico	Tobacco	L. Fucikovsky	110.2 b
245	1	Santander, Colombia	Tobacco	G. A. Granada, G2	150.6 a
272	1	Turrialba, Costa Rica	Potato	L. Sequeira	81.1 c
283	1	Quincy, FL	Soil	G. A. Granada	138.8 a
291	1	Darwin, Queensland, Australia	Tomato	A. C. Hayward	106.9 bc
273	1	Turrialba, Costa Rica	Potato	L. C. Gonzalez	79.6 c
329	1	Taiwan	Tobacco	T. Mew, T51	112.4 b
53	2	Palo Verde, Honduras	Banana	L. Sequeira, S203	106.3 bc
98	2	Costa Rica	Banana	I. W. Buddenhagen, 100	90.4 c
135	2	Buena Vista, Honduras	Banana	L. A. Berg	116.6 b
290	2	Siquirres, Costa Rica	Banana	L. C. Gonzalez, G20	81.1 c
28	3	Cyprus	Potato	W. J. Dowson	100.8 c
51	3	Gorandiyantenna, Sri Lanka	Potato	C.M.I. B2865	90.5 c
81	3	Popayan, Colombia	Potato	L. Sequeira, S207	110.4 b
82	3	Tibaitata, Colombia	Potato	H. D. Thurston	125.7 ab
119	3	Paraiso, Costa Rica	Potato	L. Sequeira, S213	41.0 d
260	3	Cajamarca, Chota, Peru	Potato	E. R. French	87.6 c
276	3	Mexico	Potato	L. Fucikovsky	115.4 b

[z] Percent plating efficiency = (avg. no. colonies detected on selective medium × 10^2)/(avg. no. colonies detected on Kelman's TZC medium). Figures represent the mean of three replicates. Any two means followed by a common letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Reduction of contaminating soil bacteria. Reduction of total soil bacteria on SM-1 was more than 99% in 11 samples from soils with populations of soil bacteria ranging from 1.02×10^7 to 1.03×10^8 cfu/g dry wt. These assays involved soils from different locations in the United States (Florida, Georgia, North Carolina, Wisconsin), where different crops (tomato, corn, onion, carrot, or potato) had been grown (Table 2). With two soil samples from Costa Rica, total bacterial counts were reduced by only 93 and 67% but *P. solanacearum* was isolated easily. These two soil samples contained high background numbers of bacteria and a few of these bacteria (not identified) were able to grow readily on SM-1.

Recovery of *P. solanacearum* from artificially infested soil. The good plating efficiency and marked reduction of background bacteria obtained with SM-1 allowed the use of low dilutions (1:5–1:100) of the soil samples, making the medium suitable for isolating the bacterium when present in relatively low populations (Fig. 1). The bacterium was recovered from soil artificially infested with populations as low as 4.4×10^2 cfu/g (Table 3). Recovery ranged from 49 to 100% when low (4.43×10^2 cfu/g) and high (3.30×10^5 cfu/g) bacterial populations, respectively, were added to soil (Table 3). Efficiency of recovery, however, varied with the strain and the type of soil.

Isolation of *P. solanacearum* from naturally infested soil. The selective medium was effective for isolating *P. solanacearum* from different types of soils from the United States and Costa Rica. Populations as low as 2.2×10^2 cfu/g dry wt and as high as 10^6 cfu/g dry wt were detected in naturally infested soils from different areas and different cropping systems (Table 4). In addition, the medium was tested in Peru (C. Martin, *personal communication*) and in Japan (H. Tanaka, *personal communication*) with good results. The susceptibility to thimerosal of some strains from

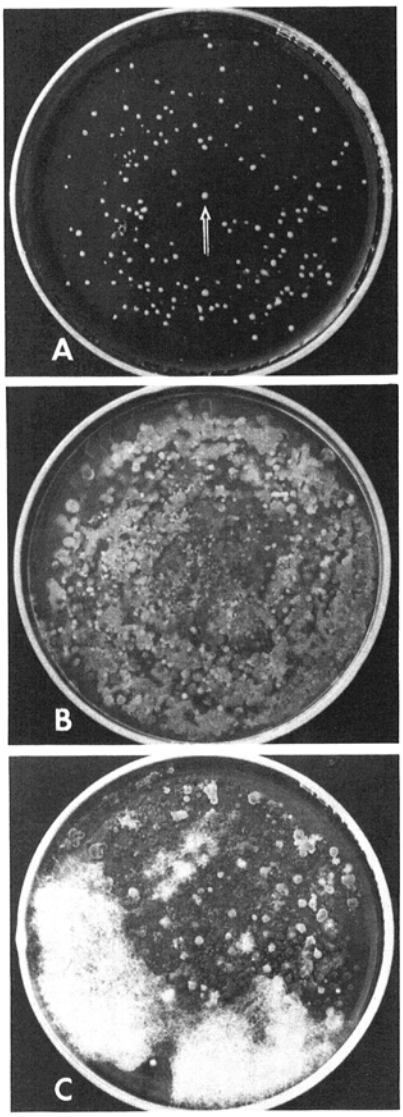

Fig. 1. Comparative efficiency of various agar media for recovery of *P. solanacearum* from artificially infested soil (10^3 cfu/g) at 1:10 dilution. **(A)** SM-1 (colonies appear milky white, indicated by the arrow), **(B)** casamino acids-peptone-glucose (CPG), and **(C)** Kelman's tetrazolium chloride medium (TZC) 3 days after plating.

Table 2. Total bacterial populations in soils from different geographic locations as determined with nonselective (CPG) and selective (SM-1) media

Location and crop	Type of soil	Total bacteria/g dry wt on: CPG[a]	Total bacteria/g dry wt on: SM-1[b]	Reduction of soil bacteria[c] (%)
Georgia (tomato)	Sandy	1.30×10^7	4.58×10^4	99.7 a
Florida (tomato)	Sandy	1.61×10^7	3.38×10^4	99.8 a
Florida (tomato)	Sandy	1.22×10^7	7.32×10^3	99.9 a
N. Carolina (tomato)	Sandy	5.85×10^6	1.75×10^4	99.7 a
Wisconsin (garden)	Loam	1.03×10^7	2.57×10^4	99.8 a
Wisconsin (onion)	Muck	2.14×10^7	1.71×10^4	99.9 a
Wisconsin (mint)	Muck	1.27×10^7	5.08×10^4	99.6 a
Wisconsin (carrot)	Muck	5.28×10^7	3.16×10^4	99.9 a
Wisconsin (pea)	Loam	1.02×10^7	1.83×10^4	99.8 a
Wisconsin (corn)	Loam	2.02×10^7	3.83×10^4	99.8 a
Costa Rica (potato)	Loam	5.57×10^8	3.84×10^7	93.1 a
Costa Rica (potato)	Loam	7.55×10^7	2.46×10^7	67.4 b
Wisconsin (potting)	Muck	1.03×10^8	2.16×10^5	99.8 a

[a] CPG = casamino acids-peptone-glucose.
[b] SM-1 = selective medium
[c] 100 − (No. bacteria recovered on SM-1/g soil) $\times 10^2$/total no. soil bacteria recovered on CPG/g soil. Figures represent the mean of three replicates. Any two means followed by a common letter are not significantly different ($P = 0.05$) after angular transformation.

Table 3. Efficiency of the selective medium (SM-1) for recovery of *Pseudomonas solanacearum* from artificially infested soils

Soil source	Type of soil[w]	Strain[x]	Race	cfu Added/g	cfu Recovered/g[y]	Recovery[z] (%)
Garden plot	Loam	25	1	3.41×10^5	1.98×10^5	58.1 c
Onion field	Muck	25	1	3.30×10^5	3.31×10^5	100.6 a
Pea field	Loam	25	1	3.30×10^5	2.72×10^5	82.4 b
Tomato field	Sandy	283	1	2.45×10^3	2.22×10^3	90.6 ab
Mint field	Muck	290	2	1.98×10^6	1.55×10^6	78.3 b
Garden plot	Loam	81	3	2.78×10^5	2.80×10^5	100.7 a
Garden plot	Loam	81	3	4.25×10^3	2.63×10^3	61.8 bc
Garden plot	Loam	81	3	4.43×10^2	2.16×10^2	48.8 c
Carrot field	Muck	81	3	3.43×10^5	3.51×10^5	102.3 a

[v] All soils from different locations in Wisconsin, except for one sample (tomato field) from Athens, GA.
[w] For background populations in the different soil samples studied, refer to Table 2.
[x] Strains of *P. solanacearum* were 25, 283 (race 1); 290 (race 2); 81 (race 3).
[y] Bacterial suspension was added to soil (1 ml/g), and after thorough mixing, samples were removed for recovery assays.
[z] Any two means followed by a common letter are not significantly different ($P = 0.05$) after angular transformation.

Table 4. Isolation of *Pseudomonas solanacearum* from naturally infested soil with the selective (SM-1) medium

Soil source	Type of soil	Locality	cfu/g Dry wt[a]
Tomato field	Sandy	Quincy, FL	3.11×10^5
Tomato field	Sandy	Athens, GA	2.20×10^2
Tomato field	Sandy	Athens, GA	1.22×10^5
Nursery	Sandy	Clinton, NC	3.62×10^2
Potato field	Loam	Turrialba, Costa Rica	6.39×10^6

[a] Each figure is the average of five replicate samples, three plates per sample.

Japan, however, required elimination of this compound from the medium.

Modifications of the selective medium. Although soil bacteria were reduced in most cases by more than 99% on SM-1 (Table 2), occasionally some bacteria from certain tropical soils grew on the medium. To remedy this problem, novobiocin (10 μg/ml) and nalidixic acid (1 μg/ml) were substituted for tyrothricin and chloromycetin, respectively. With the use of this modified medium (SM-2) background bacterial populations were reduced by more than 99% and *P. solanacearum* was recovered with high efficiency. Some strains of *P. solanacearum*, however, are susceptible to nalidixic acid; thus, prior testing of strains for susceptibility to this compound is required. Because the antifungal activity of SM-2 is lower than that of SM-1, occasionally it was necessary to add cycloheximide (50 μg/ml), especially when low soil dilutions (1:5, 1:10) were used.

DISCUSSION

Development of a selective medium for *P. solanacearum* has been difficult because different strains of this pathogen, and the microfloras of soils from different locations, differ in susceptibility to antimicrobial compounds. With the medium we devised, some of these difficulties have been reduced markedly but not eliminated. From the onset, the primary objectives in designing a new selective medium were 1) to maintain TZC as the basal medium because of the distinctive appearance of *P. solanacearum* colonies on this medium, 2) to add to TZC antimicrobial compounds that could be tested for efficiency on the principle of selective exclusion (23), and 3) to allow the use of low soil dilutions (no greater than 10^{-2}) so that low populations of the bacterium could be detected. The SM-1 medium that was devised had excellent selective characteristics for the following reasons:

1. The plating efficiency was excellent, ranging from 80 to more than 100% of that on TZC medium. When recovery was higher than on TZC, this may have resulted from surfactant characteristics of the antibiotics polymyxin and tyrothricin. The reduction in surface tension may have allowed better distribution of the bacteria on plates of selective medium as compared with TZC. Although plating efficiency beyond 100% has been reported for other selective media (6,12), this was associated with differences in nutrient composition, which was not the case for SM-1 as compared with TZC. Karganilla and Buddenhagen (9) reported that in their medium, colony counts of *P. solanacearum* were reduced by 37% as compared with TZC, but other authors have not reported the plating efficiency of their media.

2. With most soils tested, the reduction of populations of background soil bacteria on SM-1 was more than 99%. Two soil samples from Costa Rica were exceptions because background populations were reduced 93.1 and 67.4%; yet, isolation of *P. solanacearum* was easily accomplished. For other selective media, only Karganilla and Buddenhagen (9) reported a similar reduction of soil bacteria from 66 to 99%.

3. The marked reduction of contaminating soil bacteria obtained with SM-1 allowed use of low soil dilutions, thus increasing the possibility of recovering low populations of *P. solanacearum* from soil. Recovery of *P. solanacearum* was possible in many cases with soil dilutions as low as 1:4–1:5 of soils carrying 1×10^7 cfu/g soil; however, efficiency of recovery varied with the strain and the type of soil. For strain 25, for instance, at 3×10^5 cfu/g, recovery from garden loam soil was only 58%, but from muck soil, it was 100%. Recovery of strain 81 from both types of soil, however, was 100%. When dealing with very low numbers of *P. solanacearum*, the efficiency of recovery was low; thus, a large number of replicate samples had to be used to detect the bacterium.

With the selective media reported in the literature, the most efficient recovery of *P. solanacearum* was usually obtained at about 10^4 cells per gram of soil. Tanaka (20) reported recoveries ranging from 30 to 100% with Drigalski's modified medium. Nesmith and Jenkins (14) reported recoveries ranging from 51 to 92% with soils containing more than 10^4 cells per gram compared with 12–78% at 10^3 cells per gram. With Okabe's modified medium, Amat et al (1) reported recoveries as high as 97.1%. With CVP, a pectate medium developed primarily for *Erwinia* spp., recovery of *P. solanacearum* was reported as high as 89% (4).

Other favorable characteristics of the SM-1 medium are: 1) it is easy to prepare, 2) most strains of *P. solanacearum* grow well on it, 3) counts can be made after incubating the plates at 30 C for only 2–3 days, 4) the colony type is similar to that observed on TZC; therefore, the bacterium can be recognized easily, and 5) it is stable when stored as long as 3–4 wk at 4 C. Although the medium may not work well in all instances, modifications can be introduced in the original formula, as in SM-2, to best fit the medium to particular soil microflora.

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