

## Evaluation of Selective Media for Isolation of Soft-Rot Bacteria from Soil and Plant Tissue

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### ABSTRACT

A medium (CVP) containing  $\text{NaNO}_3$ , sodium polypectate, and crystal violet was compared with other selective media for the isolation of soft-rot bacteria from soil. Pectolytic colonies of *Erwinia* spp. could be distinguished from those of *Pseudomonas* spp. by the type of depressions formed in the pectate medium and colonial morphology. Recovery of *E. carotovora* and *E. atroseptica* from field soil on CVP range from 65 to 100%, depending upon soil type and the number of bacteria added to soil samples. Approximately 96% of the

natural soil bacteria (ca.  $6 \times 10^7$  cells/g dry wt soil) were eliminated on CVP. Addition of manganese sulfate (monohydrate) to CVP further reduced the soil bacterial populations without markedly lowering the percent recovery of soft-rot *Erwinia*. When CVP was used for detection of soft-rot bacteria in soil samples from cabbage, carrot, and potato fields, fluorescent pseudomonads (not soft-rot *Erwinia*) were the pectolytic gram-negative bacteria most frequently isolated.

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*Additional key words:* *Pseudomonas marginalis*, blackleg.

Selective media have been used with different degrees of success to isolate soft-rot bacteria from soil and decaying plant tissue. Direct soil plating techniques on agar media were considered by Leach (26) to be neither selective nor sensitive enough to detect small populations of soft-rot *Erwinia* that might survive the winter in soil in Minnesota; thus, he developed a potato (*Solanum tuberosum* L.) tuber-tissue assay method. Gram-negative soft-rot bacteria were isolated by a modification of Leach's method from a range of different types of soil in Scotland (22). Subsequently, Graham (11) found that the gram-negative soft-rot bacteria isolated by the tuber-tissue procedure from soil were not soft-rot *Erwinia* spp., but fluorescent pseudomonads. Other workers also have found that the use of plant tissue to determine numbers of soft-rot bacteria in soil was often neither specific nor accurate (24).

Many selective media containing pectin or sodium polypectate have been devised for detection of pectolytic soft-rot bacteria (2, 8, 18, 28, 35, 36, 38, 39, 44, 46, 49). Certain of these media, however, are unsuitable for plating large numbers of samples from soil because of inconvenient procedures, or lack of specificity. Media selective for soft-rot *Erwinia* that do not include pectic substrates have also been tested; examples are the crystal violet-bile agar medium of Patel (33, 34), the antibiotic-eosin-methylene blue medium of Segall (41), the modified Drigalski medium of Tsuyama and Sakamoto (48), and the salicin-sodium taurocholate-bromthymol blue medium of Noble and Graham (32).

More recently, Kado and Heskett (20) and Miller and Schroth (29, 30) have reported the development of media effective for the isolation of *Erwinia* spp., including the soft-rot group.

Among other techniques evaluated, an

immunofluorescent staining procedure (23) for detection of *Erwinia aroideae* (Townsend) Holland in soil was found to be unsuitable for examining large numbers of soil samples containing diverse strains of the pathogen.

In initial studies to determine the populations of soft-rot *Erwinia* in potato field soils in Wisconsin at different seasons of the year, various media tested proved to be either time-consuming in preparation or ineffective for the detection of low populations of pectolytic bacteria from soil. The objective of this investigation was to develop an improved selective medium to be used in assaying soils and plant tissues for gram-negative aerobic soft-rot bacteria, in general, and, in particular, for the *Erwinia* that rot potato tubers. A preliminary report on this study has been presented (6).

**MATERIALS AND METHODS.—Cultures.**—A strain of *Erwinia carotovora* (Jones) Holland (SR 53), originally isolated by L. R. Jones and listed by the American Type Culture Collection as type culture ATCC 495, was used throughout this work. Cultures SR 10, isolated in 1969 from rotting carrot (*Daucus carota* L.) tissue from Lake Mills, Wisconsin, and SR 8, isolated the same year from a potato plant with blackleg symptoms from Hancock, Wisconsin, were also used; these isolates had the essential characteristics of *E. carotovora* and *E. atroseptica* (van Hall) Jennison, respectively.

**Test media.**—The medium (CPG) used to grow bacterial inoculum contained (g/liter): glucose, 10; Bacto-peptone, 10; Bacto-casamino acids, 1; and Bacto-agar, 18 (21).

Initially, a pectate medium (PM) was prepared following the method of Beraha (2): (i) 1 N NaOH (4.5 ml), 10%  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$  (3 ml freshly prepared), 1.5% aqueous bromthymol blue (0.5 ml), Bacto-agar

TABLE 1. Percent reduction of soil bacteria and percent recovery of *Erwinia carotovora* (SR 53) from nonsterile field soil using various selective media

Basal medium	Additive	Concn (mg/ml)	Percent reduction of soil bacteria <sup>a</sup>	Recovery of <i>E. carotovora</i> <sup>b</sup>
Beraha			6	84
CVP <sup>c</sup>			97	79
CVP <sup>d</sup>			97	77
CVP	Yeast extract	0.1	95	93
CVP	2,3,5-triphenyl-tetrazolium chloride	0.04	82	5.6
CVP	Lithium chloride	7.0	99	0.01
CVP	Sodium azide	0.02	96	78
CVP	Sodium selenite	2.0	99	0.01
CVP	Polymyxin	1 × 10 <sup>-4</sup>	98	0.001
CVP	Bacitracin	2.0	98	1.0
CVP	Brilliant green	0.02	92	0.7
CVP	Manganese sulfate (monohydrate) <sup>e</sup>	2.5	99	79
CVP	Sodium dichromate	0.2	99	0.01
CVP	Sodium dodecylsulfate	0.05	95	70

<sup>a</sup>(% reduction of soil bacteria) =  $100 - \left( \frac{\text{no. bacteria recovered with the test medium/g soil}}{\text{total no. soil bacteria/g soil}} \times 10^2 \right)$  The average number of bacteria recovered on CPG agar from one g (dry wt) of cabbage field soil was  $6 \times 10^7$  (based on 10 separate platings).

<sup>b</sup>(% recovery of *E. carotovora*) =  $\left( \frac{\text{no. of } E. \text{ carotovora/g soil recovered with the test medium}}{\text{no. of } E. \text{ carotovora added/g of soil}} \times 10^2 \right)$

<sup>c</sup>Crystal violet was added to the medium before sterilization.

<sup>d</sup>Crystal violet was added aseptically to the medium after sterilization.

<sup>e</sup>A sterile solution of manganese sulfate was added to the surface of hardened medium in petri plates.

(1.5 g), and Bacto-yeast extract (5 g) were blended with 300 ml boiling distilled water for 15 s at high speed in a Waring Blendor, and then (ii) to this mixture 15 g sodium polypectate (Sunkist No. 6024, Orange Products Division, Sunkist Growers, Ontario, California) and 200 ml boiling distilled water were added slowly and blended. The preparation (final volume, 500 ml) was placed in a 2-liter flask, capped with aluminum foil, and autoclaved for 25 min at 120 C. The medium was poured into plates (20 ml/plate) as soon as possible after removal from the autoclave since it solidifies quickly and cannot be remelted. Final pH of the medium was 7.2

A modified pectate medium (MPM) was prepared as above except that bromthymol blue was omitted, yeast extract was reduced from 5.0 g to 0.05 g and 1.0 g NaNO<sub>3</sub> was added.

Crystal violet pectate (CVP) medium which became the major test medium was made by adding 1.0 ml of 0.075% aqueous crystal violet solution (w/v) before sterilization to 500 ml of MPM containing no yeast extract. Before being used, plates were dried at 36 C for 24-48 h.

Other test media were prepared by adding various concns of the following compounds to CPG or CVP: brilliant green (Allied Chemical Corp.), 2,3,5-triphenyltetrazolium chloride, sodium selenite, sodium azide, lithium chloride, manganese sulfate (monohydrate) (5, 15), thallium nitrate, bacitracin

(Nutritional Biochemicals), and polymyxin B sulfate (Pfizer Laboratories) (see Tables 1, 2).

The following media were prepared as described by the indicated investigators: D3 medium [Kado and Heskett (20)]; the modified Drigalski medium [Tsuyama and Sakamoto (48)]; modified Stewart's medium [Perombelon (36)]; and the Miller-Schroth medium (29, 30).

TABLE 2. Percent recovery of *Erwinia* spp. and *Pseudomonas solanacearum* from water suspensions with crystal violet pectate medium (CVP) and Miller-Schroth (MS) medium

Isolate and culture designation	Host and source	Recovery (%)	
		CVP	MS
<i>Erwinia atroseptica</i> (SR 8)	Potato in Wisconsin	76	22
<i>E. atroseptica</i> (SR 55; ICPB-EA155)	Potato in Scotland	85	17
<i>E. carotovora</i> (SR 40; ICPB-EC2)	Potato	85	54
<i>E. chrysanthemi</i> , corn pathotype (SR 80; W3-20)	Corn in Wisconsin	85	42
<i>Pseudomonas solanacearum</i> (25-K60)	Tomato in N. Carolina	89	0

Soil extract agar (1) contained, per liter of soil extract: Bacto-agar, 15 g;  $K_2HPO_4$ , 0.2 g; and Bacto-peptone, 5.0 g. This medium was adjusted to pH 7.0 before sterilization.

*General procedures for medium evaluation.*—Cultures were streaked on CPG agar and incubated at 22 C for 24 h. A water suspension of the culture (ca.  $10^8$  cells/ml) was diluted serially and surface plated directly on test media and CPG agar. Sterile distilled water was added to a 25-g soil sample from a cabbage seedbed plot in the Madison area until a total volume of 250 ml was reached. The soil suspension was shaken for 50 min on a rotary shaker (120 rpm) (19), then diluted serially, and surface plated on various test media. One ml of the water suspension of *E. carotovora* was added to another 25 g of cabbage seedbed soil and mixed thoroughly. This suspension was shaken, diluted and plated in the same manner. All plates were incubated for 3 days at 22 C.

For the various pectate test media, at least six plates per dilution were used to determine the number of bacteria per ml of suspension or per g of soil.

Evaluation of selective media for pectolytic *Erwinia* species was based on percent recovery of *Erwinia* from a water suspension in comparison with recovery from the nonsterile soil sample (cabbage seedbed), and on percent reduction of the total natural bacterial population in the nonsterile soil sample. The field soil used in most studies contained approximately  $1.2 \times 10^8$  total bacteria/g dry wt based on soil extract agar assays; the total bacterial count based on CPG agar assays was usually 50% lower.

*Procedures followed to characterize pectolytic bacteria isolated on CVP.*—Cells were removed from 24-h-old CPG agar plate cultures and stained by Hucker's modification of the Gram stain technique (43).

All polypectate-hydrolyzing isolates were tested for their ability to rot potato tubers. Tubers were immersed for 3 min in a 0.64% fresh aqueous sodium hypochlorite solution and then aseptically sliced into sections about 1.3 cm thick. Each slice was placed in a sterile petri dish on sterile, moistened filter paper. One loopful of cells from a 24-h-old CPG agar culture was placed in a freshly made slit on the upper surface of each slice. Rot production and fluorescent pigment formation were recorded if present after incubation for 48 h at 22 C. Production of cytochrome oxidase, indole, and urease was determined with PathoTec paper strips (General Diagnostics Division, Warner-Chilcott Laboratories). Urease production was also tested with Christensen's urea agar (4). Motility of the isolates was determined by microscopic examination of hanging drop suspensions made from 18- to 24-h-old CPG agar cultures. In the test for lipase formation (42), the substrate Tween 80 (polyoxyethylene sorbitan monooleate) was used. For detection of arginine dihydrolase, the Thornley method (47) was followed; however, the final pH was lowered from 7.2 to 6.8 with 1 N NaOH to enhance the sensitivity of the reaction. Gelatin liquefaction

was determined by the method of Ewing (9). In the test for casein hydrolysis, nutrient agar plates (containing 1 ml of sterile skim milk/plate) were streaked with inoculum from 24-h-old cultures and incubated for 3 days at 22 C. A positive reaction was indicated by a clear zone around the bacterial growth.

Cultures grown for 48 h at 22 C on the B medium of King et al. (25) were examined for fluorescence under ultraviolet light (366 nm).

A modification of the Hugh-Leifson oxidative-fermentative medium (17, 45) was used to determine the metabolic pathway for glucose utilization. In the test for manganese tolerance, the basal medium of Moustafa and Whittenbury (31) ( $K_2HPO_4$ , 0.008 g;  $KH_2PO_4$ , 0.002 g;  $MgSO_4 \cdot 7H_2O$ , 0.02 g; Bacto-yeast extract, 0.1 g; distilled water, 1 liter) was used. One-tenth ml of sterile 7%  $MnCl_2 \cdot 4H_2O$  solution was added to a test tube containing 7-ml of sterile medium to obtain a final concentration of 0.274 mg/ml of manganese. The pH of each tube was adjusted to 6.6 - 6.8 with 0.1 N NaOH. After incubation for 5 days at 22 C, the optical density of each bacterial suspension was measured with a Beckman DB Spectrophotometer (440 nm). A precipitate that formed in the tubes was removed by filtration with Whatman No. 2 qualitative filter paper before these measurements were taken.

Pectolytic isolates were tested for pathogenicity by inoculation of 4-wk-old potato plants (cultivar 'Russet Sebago'). Four to six plants per isolate were inoculated by puncturing the stems at the third or fourth leaf axil from the top with a toothpick tip smeared with cells from a 24-hr-old CPG plate culture of the isolate. Each puncture wound was covered with a sterile, moistened piece of absorbent cotton kept in place with Scotch tape. The plants were examined for symptoms after incubation for 7 days at 24 C.

**RESULTS.**—An improved pectate medium (CVP) was obtained by adding crystal violet to PM and using  $NaNO_3$  as the sole nitrogen source. The background population of soil bacteria usually was reduced 97% with CVP; nevertheless, the recovery of *E. carotovora* from soil was 77-79% (Table 1). Concns of crystal violet higher than 1.5  $\mu$ g/ml (i.e., 2 and 3  $\mu$ g/ml) were inhibitory for *E. carotovora* (SR 53) and *E. atroseptica* (SR 8). The Holding (14) crystal violet medium for gram-negative soil bacteria was more selective if the dye was added after sterilization. However, the results with CVP were not significantly improved by adding sterile crystal violet solution to the medium after, rather than before, sterilization.

Colonies of soft-rot *Erwinia* spp. were easily recognized on CVP by the deep cup-like depressions that they formed in the medium (Fig. 1). Depressions formed by other pectolytic soil-borne bacteria were usually shallow and wide. A similar observation had been made with another pectate medium (28). Examined under oblique light (13), soft-rot *Erwinia* colonies on CVP were iridescent, translucent, and crisscrossed with internal markings.

On D3 medium (20), recovery of SR *Erwinia* was higher from soil (14%) (Fig. 2) than from a water

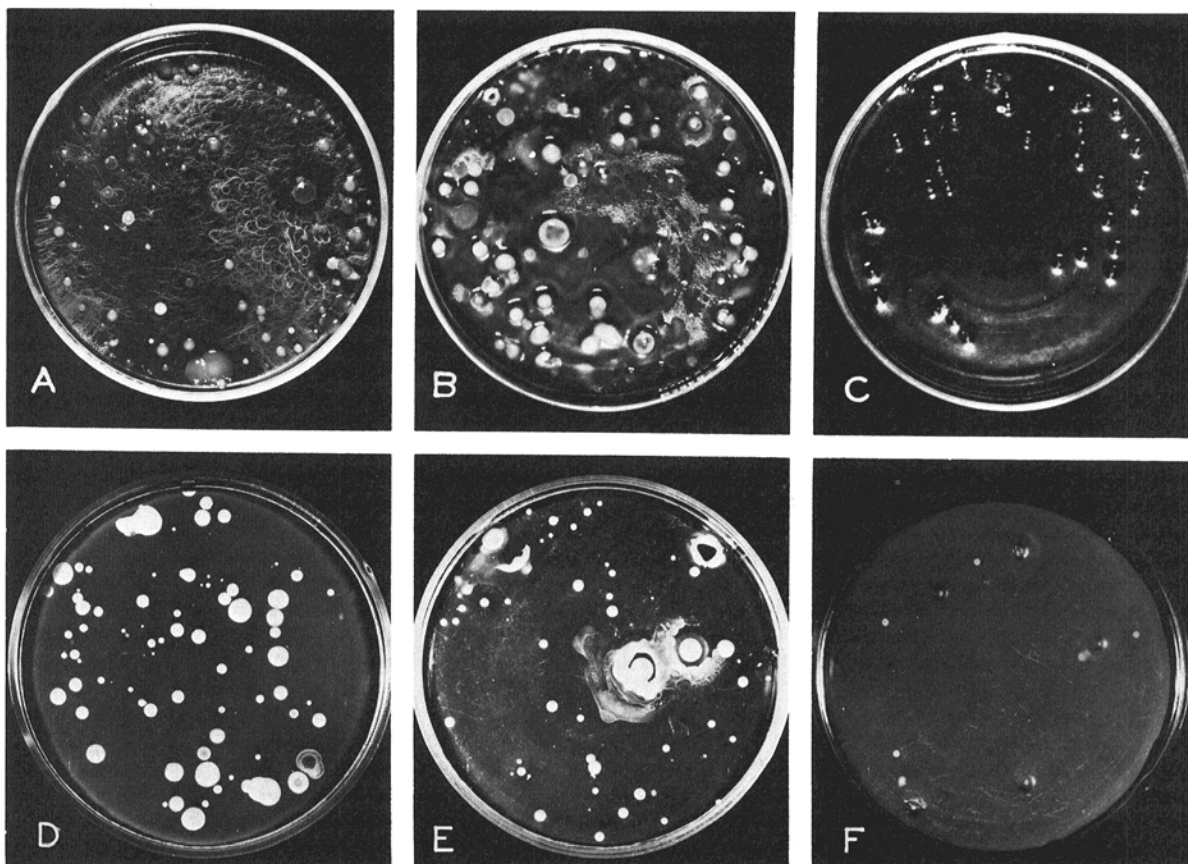


Fig. 1-(A to F). The selective ability of crystal violet pectate (CVP) medium (C and F) as compared with that of casamino acid-peptone-glucose-agar (CPG) (A and D), Beraha pectate medium (B), and modified pectate medium (E). (A to C) represent a  $10^{-5}$  dilution of cabbage field soil to which *Erwinia carotovora* had been added at  $5.6 \times 10^6$  cells/g soil; (D to F) represent a  $10^{-6}$  dilution of soil to which *E. carotovora* had been added at  $5 \times 10^6$  cells/g soil.

suspension (0.03%). On the Miller-Schroth (MS) medium (29, 30), the reverse was true. A comparison of recovery percentages obtained with CVP and MS medium for three *Erwinia* species and one *Pseudomonas* sp. from water suspensions is shown in Table 2. Compared to CVP, both D3 and MS medium gave relatively low recovery of SR *Erwinia* from soil, yet were extremely inhibitory to soil bacteria (over 99.5% reduction) (Fig. 2). The greatest differential between recovery of *Erwinia* and soil bacteria was obtained on the CVP medium. As noted by T. D. Miller (personal communication), the toxicity of MS medium for SR *Erwinia* may be attributable to the age of the poured plates (1-2 wk) used in these tests. Apparently the toxicity of this medium to SR bacteria increases with time following preparation. We have recently confirmed Miller's observations in

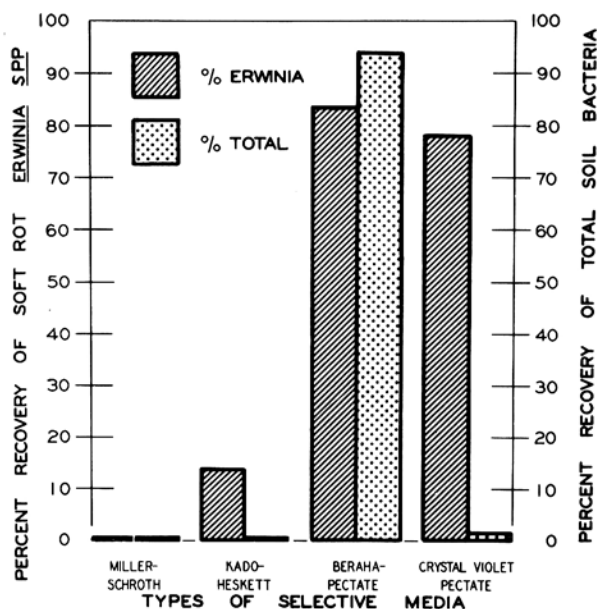


Fig. 2. Percent recovery of soft-rot *Erwinia* from soil as compared with percent recovery of soil bacteria with various selective media.

an experiment in which platings of isolates of *E. carotovora* (2), *E. atroseptica* (2), and *E. chrysanthemi* (1) were made on the MS medium on the day the medium was prepared. Recovery percentages (from water suspensions) ranged from 88-96% for four of the five isolates tested.

With both modified Drigalski medium and modified Stewart medium the percent recovery of *E. atroseptica* (SR 8) from water suspension and nonsterile soil was 90% and 77%, respectively. Modified Drigalski medium gave a higher percent reduction of soil bacteria (90%) than the modified Stewart medium (69%). On plates of modified Drigalski medium with 50 or less colonies, SR 8 formed large, bright yellow-orange colonies with a dark orange center. On plates with more than 50 colonies, however, it was indistinguishable from certain lactose-utilizing soil bacteria. *E. atroseptica* (SR 8) did not form distinctive colonies on the modified Stewart medium so the recovery rate from soil was approximated by subtracting the number of pectolytic bacteria in soil to which *E. atroseptica* had been added, from that in soil to which no bacteria had been added.

On CPG agar containing 0.04 mg/ml triphenyl tetrazolium chloride (TZC) (21), soil bacteria were reduced 60%, whereas *E. carotovora* isolates (SR 10 and SR 53) were not affected. In contrast, addition of TZC to CVP had a marked effect on *E. carotovora*; recovery from soil was only 8% and recovery from a water suspension was less than 0.1% (Table 3).

Because CVP effectively separated pectolytic and nonpectolytic bacteria, suppressed gram-positive bacteria, and was relatively nontoxic to soft-rot *Erwinia*, it was selected as the basal medium for additional tests to improve selectivity. Several

inhibitory compounds were added to CVP, but only manganese sulfate (monohydrate) reduced populations of soil bacteria without significantly affecting soft-rot *Erwinia* (Table 1).

Addition of manganese sulfate at concns above 0.8 mg/ml resulted in coagulation of the sodium polypectate during sterilization. This problem was avoided by pipetting 0.1 ml of the appropriate sterile salt solution on the surface of solid CVP medium (20 ml/plate) and distributing it over the surface with a sterile bent rod. When CVP-MnSO<sub>4</sub> medium was used to isolate bacteria from a carrot field soil, the fluorescent pectolytic pseudomonads in the total bacterial population were reduced from 21% to less than 0.1%.

Filter-sterilized thallium nitrate was added to autoclaved CVP medium to give a final concn of 0.0175 mg/ml. Recovery percentages of soft-rot *Erwinia* strains from water suspension on CVP-thallium nitrate (CVPT) medium were equivalent to those on CVP. Populations of soil bacteria in two different types of soil (clay and sandy loam) were reduced 99%, but only 53-64% of the SR 8 cells added to these soils (Table 4) could be recovered with CVPT.

The relationship of the number of *Erwinia* cells in soil to the recovery efficiency of CVP was determined by adding decreasing numbers of SR 8 to 25 g aliquots of cabbage seedbed soil (10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, and 10<sup>2</sup> cells/g dry wt soil) and then attempting to recover the microorganism by the soil isolation procedure described previously. Recovery from the soil sample to which 10<sup>6</sup>, 10<sup>5</sup>, and 10<sup>4</sup> SR 8 cells had been added was in the range of 75-80%; from the sample to which 10<sup>3</sup> SR 8 cells/g soil had been added it was 64%. When 10<sup>2</sup> SR 8 cells/g were added to soil,

TABLE 3. Comparative effectiveness of the crystal violet pectate medium (CVP) supplemented with various inhibitory compounds in the recovery of *Erwinia carotovora* (SR 53) from a water suspension containing 10<sup>7</sup> cells/ml

Inhibitory compound	Concn (mg/ml medium)	Recovery <sup>a</sup> (%)
Brilliant green	0.002	0.1
2,3,5-triphenyltetrazolium chloride	0.04	0.1
Sodium selenite	2.0	0.1
Sodium azide	0.002	80.9
Manganese sulfate	0.8	100
	1.9	100
	2.5	75
Sodium dichromate	0.2	0.1
Lithium chloride	7.0	1.0
Polymyxin B sulfate	1 × 10 <sup>-4</sup>	<0.001
	1 × 10 <sup>-3</sup>	<0.001
Bacitracin	2.0	18
Sodium dodecylsulfate	0.05	100

$$^a \left( \begin{array}{l} \% \text{ recovery of } E. \text{ carotovora} \\ \text{from water suspension} \end{array} \right) = \left( \frac{\text{no. of viable cells/ml determined with test medium}}{\text{no. of viable cells/ml determined with CVP medium}} \times 10^2 \right)$$

<sup>b</sup>The lowest concn of MnSO<sub>4</sub> · H<sub>2</sub>O was incorporated directly into the medium, but the higher concns were added as overlays to medium already poured into petri plates.

TABLE 4. Percent recovery of *Erwinia atroseptica* (SR 8) from water suspension and from soils as compared with percent reduction of soil bacteria on CVP-thallium nitrate (CVPT) medium

Source	SR 8 cells added (no.)	Recovery of SR 8 cells (%)	Reduction of soil bacteria (%)
Water suspension	$1 \times 10^7$ /ml	79	...
Clay soil	$9 \times 10^4$ /g	56	99
	$9 \times 10^3$ /g	61	99
Sandy soil	$9 \times 10^4$	64	99
	$9 \times 10^3$	53	99

the background populations of soil bacteria overgrew and obscured SR 8. The same difficulty occurred when CVPT was used as the isolation medium.

CVP was employed to determine the number of pectolytic bacteria, particularly soft-rot *Erwinia*, in the water of a washing tank at a large potato chip plant in Madison. Washwater samples were taken after approximately 10 tons of potatoes originating from one grower from a particular state had been passed through the washing tank. The water samples were then serially diluted and the dilutions plated on CPG and CVP. Calculated by the formula

$$\frac{\text{pectolytic bacteria per ml (determined with CVP medium)}}{\text{total bacteria per ml (determined with CPG medium)}} \times 10^2,$$

the percentages of pectolytic bacteria in the total bacterial populations that could grow on CVP were:

Florida, 1.0%; Alabama, 0.8%; Michigan, 0.68%; and North Dakota, 0.25%. About 40% of the pectolytic bacteria present in the washwater sample from a shipment of freshly harvested Florida potatoes were soft-rot *Erwinia* ( $8 \times 10^4$  cells/ml). The tubers in this shipment were in excellent condition, with no evidence of bacterial soft-rot, however.

Populations of soft-rot *Erwinia* in washwater or soil samples declined during storage of the samples at 4 C. The viable count (on CPG) of soil bacteria in samples taken from 1.81-2.27 kg (4-5 lb) of cabbage seedbed soil before and after 3-mo storage in a closed plastic bag was  $4 \times 10^7$  and  $5 \times 10^7$  cells/g dry weight soil, respectively. In contrast, *E. carotovora*, which initially numbered  $3.5 \times 10^6$  cells/g soil (determined on CVP medium), could not be detected after this storage period.

Five field soils in Wisconsin were assayed for the presence of pectolytic bacteria using the CVP medium (Table 5). In muck soil from a field in which an entire crop of carrots had been abandoned in 1969 due to soft-rot, pectolytic bacteria in samples taken in the early spring of 1970 numbered  $2.2 \times 10^9$  cells/g dry weight of soil. Pseudomonads capable of causing soft-rot on potato tuber slices predominated; no soft-rot *Erwinia* were found. Soil samples taken from two cabbage plots in the Madison area had considerably smaller numbers of total and pectolytic bacteria than those observed in this muck soil. Soil samples were taken during the growing season from areas of a potato field in which ca. 20-30% of the plants had blackleg caused by *E. atroseptica*. Approximately six times as many pectolytic bacteria were present in soil samples from blackleg areas, than from areas in which all plants were healthy. However, none of the colonies from either area resembled those of soft-rot *Erwinia*.

TABLE 5. Occurrence of pectolytic bacteria in field soils in which bacterial soft-rot had been observed the previous year

Source of soil sample	Bacterial populations ( $\times 10^6$ cells/g dry wt of soil)			Pectolytic bacteria in the gram-negative population <sup>a</sup> (%)
	CPG Total bacteria	CVP Total bacteria      Pectolytic bacteria		
Carrot field <sup>b</sup>	6240	2240	1320	58.9
Cabbage fields				
Plot A	92	14.7	0.04	0.27
Plot B	203	27.7	0.45	1.6
Potato field <sup>c</sup>				
Soil A	240	10.3	0.38	4.7
Soil B	150	2.4	0.77	26.4

$$^a \left( \frac{\% \text{ pectolytic bacteria}}{\text{in total soil bacteria}} \right) = \left( \frac{\text{no. of pectolytic bacteria/g soil as determined on CVP}}{\text{total number of bacteria/g soil as determined on CVP}} \times 10^2 \right)$$

<sup>b</sup>The entire crop of carrots in this muck soil field was not harvested because of adverse weather conditions and severe soft-rot incidence in August, 1969. A soil sample was taken in April, 1970 from an area in the field where severe rotting of carrots had occurred.

<sup>c</sup>Random soil samples were taken in December, 1970 from a potato field with large numbers of plants with blackleg symptoms. Soil A came from an area of the field where little blackleg had occurred; soil B came from an area where blackleg was prevalent.

Pectolytic bacteria that formed translucent colonies with shallow pits on CVP were isolated from cabbage and carrot field soils and compared with *E. atroseptica* (SR 8). All 48 isolates produced a rapid soft-rot of potato slices and were identified as fluorescent pseudomonads. Tests for casein hydrolysis and production of arginine dihydrolase, gelatinase, urease, and lysine decarboxylase were positive for 13 of these pseudomonad isolates selected at random. These isolates did not produce lipase and were inhibited by manganese.

Although able to cause soft-rot of potato slices, the pseudomonads could not produce, in greenhouse inoculations, the stem blackening and eventual collapse of potato plants typical of the blackleg disease caused by *E. atroseptica* at 24 C. Symptoms of infection by *Pseudomonas* spp. isolates were a browning and slight softening of the tissue around the site of inoculation. With respect to the characteristics studied, these *Pseudomonas* isolates appear similar to *P. marginalis* (Brown) Stapp (7, 27).

All soft-rot *Erwinia* cultures tested (seven *E. atroseptica* isolates, one pathotype of *E. chrysanthemi* from corn, one *E. aroideae* isolate, and two *E. carotovora* isolates) were not inhibited by manganese at the concns tested.

DISCUSSION.—Quantitative expression of the effectiveness of a selective medium in isolations from such a complex source as field soil can be only an approximation. Nevertheless, the methods employed in this study provided the basis for evaluating various test media for detection of soft-rot *Erwinia*. The CVP medium that was developed had certain advantages over other media tested. The merit of CVP lies in its ability to suppress a substantial percentage of the bacterial populations in soil, its simple method of preparation, and the distinctive appearance of colonies of soft-rot *Erwinia* on its surface. Furthermore, it is not as inhibitory for soft-rot *Erwinia* as are other selective media that have been tested. Although gram-positive bacteria, which often represent approximately 93% of the bacterial population of soil outside the rhizosphere of plants (14, 15), grow poorly or not at all on CVP, this medium does support the growth of certain nonpectolytic gram-negative bacteria. The latter group, whose numbers vary with the soil source (Table 3), can obscure the growth of soft-rot *Erwinia* only if the number of *Erwinia* in the soil population is very low. Even under these conditions *Erwinia* spp. colonies are still detectable by the characteristically deep depression they form in the medium. Even when the ratio of *Erwinia* spp. to soil bacteria was 1:500,000, these depressions could be observed. However, no definite counts can be made and each colony would require isolation and a secondary plating on CVP to confirm its identification. Addition of thallium nitrate to CVP reduced the number of soil bacteria by an additional factor of 10, but CVPT, too, cannot be used efficiently when the *Erwinia* to soil bacteria ratio is low.

Although the addition of manganese sulfate to CVP effectively reduced the pseudomonad

population in soil, its use in large-scale surveys would be impractical because of the time-consuming method needed for its application.

Our initial observations indicate that soft-rot *Erwinia* are not well adapted to survival in nonsterile soil held at near freezing temp. These data and the observations by investigators in Scotland (11, 12) and Ireland (28) indicate the need to reassess the concept that blackleg-causing and related *Erwinia* spp. can survive winter conditions in the North Central region of the United States (26). In contrast, soft-rot pseudomonads, such as those found in the carrot field, seem to be able to endure the severe Wisconsin winters. These particular soft-rot pseudomonads were easily recognized by their colonial morphology and the type of pits formed on CVP. The occurrence of fluorescent pectolytic pseudomonads in soil, on market vegetables, and in cases of storage rot has been noted before (3, 10, 11, 16, 35, 40). Most of the strains isolated in this study have many characteristics in common with *P. marginalis* (Brown) Stapp.

Platings from soil with CVP have advantages over the use of plant tissue to detect small populations of soft-rot *Erwinia* because of the nonselectivity of the latter method and the ubiquity of other soft-rotting bacteria, particularly *Bacillus* spp. and fluorescent pseudomonads. A recently developed method involving incubation of the tubers under anaerobic conditions and high humidity has been found by Perombelon (37) to be effective in detection of the presence of soft-rot bacteria in lenticels of ostensibly healthy potato tubers. This method does require isolation from infected tissue for diagnosis of the specific causal bacterium. The CVP medium has been utilized to advantage in our laboratory for this purpose as well.

#### LITERATURE CITED

1. ALLEN, O. N. 1957. Experiments in soil bacteriology. 3rd rev. ed. Burgess Publishing Co., Minneapolis, Minn. 117 p.
2. BERAHA, L. 1968. A rapid method for the preparation of semi-solid agar medium for detection of pectolytic enzyme activity in *Erwinia carotovora*. Plant Dis. Rep. 52:167.
3. BONDE, R. 1939. Comparative studies of the bacteria associated with potato blackleg and seedpiece decay. Phytopathology 29:831-851.
4. CHRISTENSEN, W. B. 1946. Urea decomposition as a means of differentiating *Proteus* and *Paracolon* cultures from each other and from *Salmonella* and *Shigella*. J. Bact. 52:461-466.
5. CLARK, A. G. 1969. A selective medium for the isolation of *Agrobacterium* species. J. Appl. Bact. 32:348-351.
6. CUPPELS, D. A., and A. KELMAN. 1971. A selective medium for isolation of pectolytic soft-rot bacteria from soil. Phytopathology 61:1022 (Abstr.).
7. DOWSON, W. J. 1941. The identification of the bacteria commonly causing soft rot in plants. Ann. Appl. Biol. 28:102-106.
8. ELROD, R. P. 1942. The *Erwinia*-coliform relationship. J. Bact. 44:433-440.
9. EWING, W. H. 1962. Enterobacteriaceae, Biochemical Methods for Group Differentiation. U.S. Pub. Health Ser. Publ. 734. 32 p.

10. FOLSOM, D., and B. A. FRIEDMAN. 1959. *Pseudomonas fluorescens* in relation to certain diseases of potato tubers in Maine. *Am. Potato J.* 36:90-97.
11. GRAHAM, D. C. 1958. Occurrence of soft-rot bacteria in Scottish soils. *Nature (Lond)* 181:61.
12. GRAHAM, D. C., and P. C. HARPER. 1966. Effect of inorganic fertilizers on the incidence of potato blackleg disease. *Eur. Potato J.* 9:141-145.
13. HENRY, B. S. 1933. Dissociation in the genus *Brucella*. *J. Infect. Dis.* 52:374-402.
14. HOLDING, A. J. 1960. The properties and classification of the predominant gram-negative bacteria occurring in soil. *J. Appl. Bact.* 23:515-525.
15. HOLDING, A. J., and D. C. JEFFREY. 1968. Effects of metallic ions on soil bacteria. Pages 516-530. *in* T. R. G. Gray, and D. Parkinson, eds. *The ecology of soil bacteria, an international symposium.* University of Toronto Press, Canada.
16. HUETHER, J. P., and G. A. MC INTYRE. 1969. Pectic enzyme production by two strains of *Pseudomonas fluorescens* associated with the pinkeye disease of potato tubers. *Am. Potato J.* 46:414-423.
17. HUGH, R., and E. LEIFSON. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. *J. Bact.* 66:24-26.
18. JACOBELLI, G. 1953. Utilisation de milieux de culture gelifies par des substances pectiques. *C. R. Acad. Sci. Agric. Bulg.* 236:228-230.
19. JOHNSON, L. R., E. A. CURL, J. H. BOND, and H. A. FRIBOURG. 1959. Methods for studying soil microflora-plant disease relationships. Burgess Publishing Co., Minneapolis, Minn. 178 p.
20. KADO, C. I., and M. G. HESKETT. 1970. Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. *Phytopathology* 60:969-976.
21. KELMAN, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. *Phytopathology* 44:693-695.
22. KERR, A. 1953. A method of isolating soft-rotting bacteria from soils. *Nature (Lond.)* 172:1155.
23. KIKUMOTO, T., and M. SAKAMOTO. 1967. Ecological studies on the soft-rot bacteria of vegetables III. Application of immunofluorescent staining for the detection and the counting of *Erwinia ardoideae* in soil. *Ann. Phytopathol. Soc. Jap.* 33:181-186.
24. KIKUMOTO, T., and M. SAKAMOTO. 1968. Ecological studies on the soft-rot bacteria of vegetables VIII. Reliability of a carrot slice method for estimating the soft-rot bacteria in soils. *Bull. Inst. Agric. Res., Tohoku Univ.* 20:37-56.
25. KING, E. O., M. K. WARD, and D. E. RANEY. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
26. LEACH, J. G. 1931. Blackleg disease on potatoes in Minnesota. *Minn. Agric. Exp. Stn. Tech. Bull.* 76:1-36.
27. LELLIOTT, R. A., E. BILLING, and A. C. HAYWARD. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. *J. Appl. Bact.* 29:470-489.
28. LOGAN, C. 1963. A selective medium for the isolation of soft-rot coliforms from soil. *Nature (Lond)* 199:623.
29. MILLER, T. D., and M. N. SCHROTH. 1970. Selective medium for the isolation of *Erwinia carotovora* and other *Erwinia* spp. *Phytopathology* 60:1304 (Abstr.).
30. MILLER, T. D., and M. N. SCHROTH. 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. *Phytopathology* 62:1175-1182.
31. MOUSTAFA, F. A., and R. WHITTENBURY. 1970. A comparison of some phytopathogenic and nonphytopathogenic pseudomonads. *Phytopathol. Z.* 67:63-72.
32. NOBLE, M., and D. C. GRAHAM. 1956. A selective medium for the isolation of coliform soft-rot bacteria from plant tissue. *Nature (Lond)* 178:1479-1480.
33. PATEL, M. K. 1926. An improved method of isolating *Pseudomonas tumefaciens* Sm. and Town. *Phytopathology* 16:577 (Abstr.).
34. PATEL, M. K. 1929. Viability of certain plant pathogens in soil. *Phytopathology* 19:295-300.
35. PATON, A. M. 1958. Pectin-decomposing strains of *Pseudomonas*. *Nature (Lond)* 181:61-62.
36. PEROMBELON, M. C. M. 1971. A semi-selective medium for estimating population densities of pectolytic *Erwinia* spp. in soil and in plant material. *Potato Res.* 14:158-160.
37. PEROMBELON, M. C. M. 1972. A reliable and rapid method for detecting contamination of potato tubers by *Erwinia carotovora*. *Plant Dis. Rep.* 56:552-554.
38. RUDD JONES, D. 1946. A medium for investigating the breakdown of pectin by bacteria. *Nature (Lond)* 158:625.
39. SABET, K. A., and W. J. DOWSON. 1951. Action of phytopathogenic bacteria on pectate gel. *Nature (Lond)* 168:605.
40. SANDS, D. C., L. HANKIN, and M. ZUCKER. 1972. A selective medium for pectolytic fluorescent pseudomonads. *Phytopathology* 62:998-1000.
41. SEGALL, R. H. 1971. Selective medium for enumerating *Erwinia* species commonly found in vegetable packing house waters. *Phytopathology* 61:425-426.
42. SIERRA, G. 1957. A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie von Leeuwenhoek* 23:15-22.
43. SOCIETY OF AMERICAN BACTERIOLOGISTS, COMMITTEE ON BACTERIOLOGICAL TECHNIC. 1957. *Manual of microbiological methods.* McGraw-Hill, New York. 315 p.
44. STEWART, D. J. 1962. A selective-diagnostic medium for the isolation of pectinolytic organisms in the Enterobacteriaceae. *Nature (Lond)* 195:1023.
45. SUBCOMMITTEE ON TAXONOMY OF STAPHYLOCOCCUS AND MICROCOCCUS. 1965. Recommendations. *Int. Bull. Bact. Nomen. Taxon.* 15:109-110.
46. THORNE, S. N. 1972. A pectate gel for the isolation and diagnosis of pectinolytic bacteria. *J. Appl. Bact.* 35:357-358.
47. THORNLEY, M. J. 1960. The differentiation of *Pseudomonas* from other gram-negative bacteria on the basis of arginine metabolism. *J. Appl. Bact.* 23:37-52.
48. TSUYAMA, H., and M. SAKAMOTO. 1952. Isolation methods of the soft-rot causing bacteria from the soil. *Sci. Rep. Res. Inst. Tohoku Univ., Sér. D, Biol.* 3:29-34.
49. WIERINGA, K. T. 1947. A method for isolating and counting pectolytic microbes. *Proc. 4th Int. Congr. Microbiol. (Copenhagen)* 4:482-483.