A Starch-Methionine Medium for Isolation of *Xanthomonas campestris* pv. *campestris* from Plant Debris in Soil

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ABSTRACT

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A defined medium was developed for isolation of *Xanthomonas campestris* pv. *campestris* from infected cabbage leaves and plant debris. The medium, based on the minimal nutritional requirements for the genus *Xanthomonas* and Schaad and White's SX medium, contained (per liter) soluble potato starch (10 g), glucose (1 g), D-methionine (0.2 g), KH_2PO_4 (1 g), Na_2HPO_4 (2.6 g), NaCl (2 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $CaCl_2 \cdot 2H_2O$ (0.067 g), trace elements (0.02–1.0 mg), methyl violet 2B (1 ml of a 1% solution in 20% ethanol), methyl green (2 ml of a 1% aqueous solution), triphenyltetrazolium chloride (1 ml of a 1% aqueous solution), cycloheximide (50 mg), and agar (20 g). The pH was adjusted to 7.0 before autoclaving; final pH was 6.8. Recovery and recognition of *X. campestris* pv. *campestris* was improved over SX. The medium is useful for isolation of several other pathovars of *X. campestris* in addition to the black rot pathogen.

Black rot of crucifers, caused by *Xanthomonas campestris* pv. *campestris*, is a major disease of cabbage in Hawaii, where the disease apparently is perpetuated in successive crops by plant debris. The effects of edaphic factors on decomposition of plant debris and survival of the pathogen have been implicated (1) but are difficult to evaluate quantitatively because high populations of saprophytes interfere with recognition of pathogen on semiselective SX medium (9) and modified SX (2) and because recovery is poor on D5 medium (4).

The purpose of this work was to develop a defined medium for *X. campestris* pv. *campestris* with improved sensitivity and to evaluate its usefulness in isolating the black rot pathogen from infected leaf tissue in soil. In addition, the selectivity of the medium for other pathovars of *X. campestris* and other genera was assessed.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Cultures were examined for uniformity on Kelman's (5) tetrazolium medium (TZC) without casein hydrolysate. This medium is nonselective, but it aids in differentiation of *Xanthomonas* from other genera of bacteria.

Semiselective medium. A semiselective medium for isolation of *X. campestris* pv. *campestris* was developed based on nutritional requirements determined in previous studies (3,9,11). Stock solutions were prepared separately and added to the final medium in varying amounts to compare effects of growth factors, inhibitors, and pH. Phosphate buffer was a mixture of equimolar quantities of KH_2PO_4 and Na_2HPO_4 diluted to 1 M with respect to phosphate. The medium was buffered at 0.025 or 0.05 M by adding 25 or 50 ml/L, respectively. $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ were prepared in 100X stock solutions and added at 10 ml/L to final concentrations of 20 and 6.7 mg/100 ml, respectively. The trace elements solution modified from those used by Stanier et al (10) and Starr (11) contained ethylenediaminetetraacetic acid (250 mg), $FeSO_4 \cdot 7H_2O$ (500 mg), $ZnSO_4 \cdot 7H_2O$ (10 mg), $CuSO_4 \cdot 5H_2O$ (10 mg), $MnSO_4 \cdot H_2O$ (10 mg), $NaMoO_4 \cdot 2H_2O$ (25 mg), $Na_2B_4O_7 \cdot 10H_2O$ (18 mg), $CoSO_4 \cdot 7H_2O$ (10 mg), and distilled water (100 ml). Growth factors (methionine and glutamic acid), antibiotics, and triphenyltetrazolium chloride were prepared separately and added to the medium after autoclaving. Dyes used in other media for *Xanthomonas* (7,9) were compared for inhibitory effects on soil microorganisms. A starch-methionine (SM) medium was selected for further studies because *X. campestris* pv. *campestris* pathovars grew best on this combination.

Colony characteristics and media selectivity. Bacterial strains were dilution streaked onto SM medium and incubated at 28 C. Colony characteristics were observed during a 10-day period. Selectivity of the medium was evaluated by dilution plating of several pathovars of *X. campestris*. Cultures were grown for 2 days on TZC before washing in sterile distilled water (SDW) and resuspending in SDW to $A_{600} = 0.5$ (2×10^9 cells per milliliter). One-tenth milliliter of the diluted cell suspensions was spread on duplicate plates of TZC, SX, and SM. Colonies were counted 3–7 days later. Other genera of bacteria were dilution streaked on the SM medium.

Single 4-mm-diameter disks from 150 healthy and infected fresh cabbage leaves were placed into 2 ml of sterile water and extracted overnight. The extracts were assayed by dilution streaking on TZC and SM and by injecting 1-ml aliquots into interveinal areas of susceptible cabbage leaves for pathogenicity tests (C-G hybrid of head cabbage, Takii and Co., Kyoto, Japan). Recovery by the assay method was evaluated using 75 healthy and 75 diseased samples determined by visual symptoms.

Isolation of *X. campestris* pv. *campestris* from dried infected leaf tissue. Naturally infected head cabbage leaves collected from four farms were dried and ground in a Wiley mill to pass a 1-mm-diameter sieve. Each sample was subdivided into two subsamples. One-tenth gram of dried leaf material was added to 10 ml of SDW and extracted for 2 hr at room temperature. The tubes were then serially diluted and plated on media. In initial assays on TZC (six replicates per subsample), 1×10^7 to 8×10^8 colony-forming units (cfu) per gram were recovered.

Soil was infested by adding 1.0, 0.1, or 0.01 g of ground dried tissue from infected cabbage leaves to 10 g of unsterile soil. The infested soil (1 g) was then extracted for 2 hr in 10 ml of SDW serially diluted and plated on TZC, SX, and SM.

RESULTS

Media were compared for most rapid growth, uniformity of *Xanthomonas* colonies, differentiation from nontarget organisms, selectivity against saprophytes, and high efficiency of plating. The medium that best satisfied these criteria for recovery of *X. campestris* pv. *campestris* was SM medium, which contained potato starch (10 g), glucose (1 g), D-methionine (0.2 g), KH_2PO_4 (1 g), Na_2HPO_4 (2.6 g), NH_4Cl (1 g), NaCl (2 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $CaCl_2 \cdot 2H_2O$ (0.067 g), trace elements solution (described in Methods) (1 ml/L), methyl violet 2B (1 ml of a 1% solution in 20% ethanol), methyl green (2 ml of a 1% aqueous solution), dyes obtained from Matheson Coleman and Bell (Norwood, OH 45212), cycloheximide (0.050 g), triphenyltetrazolium chloride (1 ml of a 1% aqueous solution), and agar (20 g) per liter glass distilled water. All components except starch, cycloheximide, tetrazolium chloride, and agar were dissolved in about 800 ml of water. The pH was 7.0 before heating the medium to boiling. Starch was suspended in about 200 ml of water and added to the boiling medium. Agar was added, the total volume adjusted to 1 L, and the medium was autoclaved at 121 C for 15 min. Tetrazolium chloride was autoclaved separately at 121 C for 5 min. Cycloheximide (50 mg) and tetrazolium chloride (1 ml) were added after cooling the medium to 50 C. Final pH was 6.8.

All *X. campestris* strains that grew on SM medium were visible after incubation for 3–4 days. Colonies were circular, convex, and bluish white with scarlet red centers. After 5–7 days of growth, different colony morphologies were observed for different strains. Large mucoid colonies with dark red centers were common for *X. campestris* pv. *campestris* from cabbage (A249-1, EEXC114, EEXC118, KC 3-1-12, and OK_2) and from radish (RR 68). One isolate (A342) from broccoli was flatter with a darker center. Begonia strains (A915 and QR 30) appeared blue-green. *X. campestris* pv. *phaseoli* cultures (A602-1s and A868-2) and cultures from onion (A88-3 and A611), *Cordyline terminalis* (A765-2 and A910-2), Epipremnum (A858-2), poinsettia (A912-1), and anthurium (A774-1 and B61A) were similar to *X. campestris* pv. *campestris* isolates.

Strong zones of starch hydrolysis were observed within 3–4 days for all the strains tested except a few from anthurium, lettuce, pepper, and rice. Weak zones of starch hydrolysis were observed after 7 days for strains from anthurium (A844-1, B29, B41) and lettuce (QR 71 and 10 TB10-1). Large clear zones were observed with isolates from pepper (A777-1 and A913-1) and from rice (BU 26), where the streak was heaviest but not around single colonies.

High plating efficiencies were observed for most of the *Xanthomonas* strains tested (Table 2). Plating efficiency, defined as (avg. no. cfu on SX or SM plate)/(avg. no. cfu on TZC plate) × 100, on SM ranged from 24 to 104% for *X. campestris* pv. *campestris*. Plating efficiencies were low for *X. campestris* pv. *begoniae* (19%), *X. campestris* pv. *manihotis* (2%), *X. campestris* pv. *oryzae* (10%), and *X. campestris* pv. *poinsettiicola* (10%). No colonies of *X. campestris* pv. *vesicatoria* from pepper were observed after 10 days. Plating efficiencies on SX medium were less than 14% for all strains tested except for A249-1, which was 48%.

Table 1. Bacterial strains used to evaluate the medium SM (starch-methionine)

Strain	Laboratory designation	Origin	Source[a]
Bacillus sp.	A890	Tomato	1
Corynebacterium insidiosum	QR 80	Alfalfa	2
C. michiganense	A518-1	Tomato	1
C. sepedonicum	QR 83	Potato	2
Enterobacter aerogenes	A698	Soil	4
Erwinia carotovora subsp. *atroseptica*	QR 10	Potato	7
E. carotovora subsp. *carotovora*	QR 11	Mexican pepper	7
E. chrysanthemi	QR 12	Carnation	7
E. herbicola	QR 13	Apple	7
Escherichia coli	A699	Soil	4
Pseudomonas fluorescens	A404	Soil	1
P. syringae pv. *phaseolicola*	QR 67	Bean	6
Xanthomonas campestris pv. *begoniae*	A915	Begonia	1
	QR 30	Begonia	7
Xanthomonas campestris pv. *campestris*	A249-1	Cabbage	1
	OK_2	Cabbage	9
	KC 3-1-12	Cabbage	1
	EEXC114	Cabbage	3
	EEXC118	Cabbage	3
	PHW 46	Cabbage	8
	RR 68	Radish	8
	A342	Broccoli	1
X. campestris pv. *dieffenbachiae*	A774-1	Anthurium	1
	A844-1	Anthurium	1
	A858-2	Epipremnum	1
	A925-6	Syngonium	1
	B29	Anthurium	5
	B41	Anthurium	5
	B61A	Anthurium	5
X. campestris pv. *manihotis*	QR 32	Cassava	7
X. campestris pv. *oryzae*	BU 26	Rice	1
X. campestris pv. *phaseoli*	A602-1s	Bean	1
	A868-2	Bean	1
X. campestris pv. *poinsettiicola*	A912-1	Poinsettia	1
X. campestris pv. *vesicatoria*	A777-1	Pepper	1
	A913-1	Pepper	1
X. campestris pv. *vitians*	QR 33	Syngonium	7
	QR 71	Lettuce	7
	10 TB 10-1	Lettuce	1
Xanthomonas sp.	A88-3	Onion	1
	A611	Onion	1
	A765-2	*Cordyline terminalis*	1
	A910-2	*C. terminalis*	1

[a] 1 = Original isolate; 2 = American Type Culture Collection, Rockville MD 20852; 3 = E. Echandi, North Carolina State University, Raleigh 27607; 4 = L. R. Berger, Department of Microbiology, University of Hawaii, Honolulu 96822; 5 = M. Aragaki, Department of Plant Pathology, University of Hawaii, Honolulu 96822; 6 = S. S. Patil, Department of Plant Pathology, University of Hawaii, Honolulu 96822; 7 = M. P. Starr, International Collection of Phytopathogenic Bacteria, University of California, Davis 95616; 8 = P. H. Williams, Department of Plant Pathology, University of Wisconsin, Madison 53706; and 9 = M. Goto, Laboratory of Plant Pathology, Shizuoka University, Shizuoka 420, Japan.

Of the other bacterial genera tested on SM, only *Enterobacter aerogenes* grew well but no zones of starch hydrolysis occurred. *Erwinia carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora*, *E. herbicola*, *Pseudomonas fluorescens*, and *P. syringae* pv. *phaseolicola* also grew on SM medium but colonies were small (less than 2 mm) and no starch hydrolysis was observed after 10 days. The remaining genera tested did not grow.

The pathogen was detected on SM medium in 92% of the disks from tissue showing clear black rot symptoms and 8% of the disks sampled from symptomless tissue. Comparisons with recovery on TZC medium and production of symptoms via pathogenicity tests are presented in Table 3.

Recognition of *X. campestris* pv. *campestris* was obscured by rapid overgrowth of saprophytes when dried infected leaf material was plated on TZC, whereas lower proportions of saprophytes and clear zones of starch hydrolysis around *Xanthomonas* colonies facilitated enumeration on SX and SM media (Table 4). Recovery of the pathogen was greater on SM than SX medium.

Recovery of *X. campestris* pv. *campestris* from infested soil was improved on SM medium compared with TZC and SX media (Table 5). Both SM and SX media reduced the number of saprophytes compared with TZC.

Table 2. Plate counts and plating efficiencies for the *Xanthomonas* strains tested

Strain	Laboratory designation	Colonies per plate[a]			Plating efficiency[b]	
		TZC	SX	SM	SX	SM
Xanthomonas campestris pv. *begoniae*	A915	261	0	49	0	19
	QR 30	497	6	96	1	19
X. campestris pv. *campestris*	A249-1	391	188	311	48	80
	OK_2	349	48	364	14	104
	KC 3-1-12	374	6	339	1	91
	EEXC114	287	11	245	4	85
	EEXC118	216	26	196	12	91
	PHW 46	305	15	237	5	78
	RR 68	167	10	41	6	24
	A342	184	6	137	3	75
X. campestris pv. *dieffenbachiae*	A774-1	177	1	77	0	43
	A844-1	635	0	520	0	82
	A858-2	399	6	336	2	84
	A925-6	875	0	566	0	65
	B29	146	0	118	0	81
	B41	1,098	2	843	0	77
	B61A	253	0	153	0	61
X. campestris pv. *manihotis*	QR 32	275	0	6	0	2
X. campestris pv. *oryzae*	BU 26	356	0	37	0	10
X. campestris pv. *phaseoli*	A602-1s	470	0	27	0	6
	A868-2	339	3	150	1	44
X. campestris pv. *poinsettiicola*	A912-1	301	6	31	2	10
X. campestris pv. *vesicatoria*	A777-1	726	0	0	0	0
	A913-1	279	0	0	0	0
X. campestris pv. *vitians*	QR 33	238	24	78	10	33
	QR 71	283	2	239	1	84
	10 TB 10-1	393	7	331	2	84
Xanthomonas sp.						
From onion	A88-3	455	0	241	0	53
From onion	A611	151	0	72	0	47
From *Cordyline terminalis*	A765-2	219	0	61	0	28
From *C. terminalis*	A910-2	281	0	51	0	18

[a] Plate counts adjusted to the 10^{-7} dilution. TZC = tetrazolium chloride medium; SX = Schaad and White's selective medium for *X. campestris* pv. *campestris*; SM = starch-methionine medium for *Xanthomonas*.

[b] Plating efficiency = $\frac{\text{Avg. no. cfu on SX or SM plate}}{\text{Avg. no. cfu on TZC plate}} \times 100$.

Table 3. Comparison of culture media and pathogenicity tests in recovery of *Xanthomonas campestris* pv. *campestris* from leaves of field cabbage showing black rot symptoms

Sample material[a]	Percentage of disks positive for *Xanthomonas*		
	TZC	SM	Pathogenicity
Symptomless tissue	2.7	8.0	4.0
Tissue with black rot symptoms	84.0	92.0	90.7

[a] Fresh cabbage leaves were sampled from symptomless areas (75 4-mm leaf disks) and typical black rot V-shaped lesions (75 4-mm leaf disks). Disks were extracted and assayed as described in Materials and Methods.

Table 4. Comparison of media for recovery of *Xanthomonas campestris* pv. *campestris* from infected leaf material

Medium	Sample and subsample[a]							
	A		B		C		D	
	1	2	1	2	1	2	1	2
TZC	6/81[b]	12/78	44/83	48/70	65/73	75/82	47/66	50/65
SX	3/40	3/31	2/3	9/12	11/12	8/9	2/3	1/1
SM	89/114	98/124	19/20	21/21	37/37	32/33	27/27	24/24

[a] Naturally infected cabbage leaf material collected from four separate farms was dried and ground; subsamples (0.1 g) were extracted for 2 hr in 10 ml SDW. After serial dilution, 0.1 ml was plated on respective media.

[b] Numbers = *Xanthomonas* colony-forming units (cfu)/total cfu. Average of two replicates for each subsample.

DISCUSSION

The SM medium is a defined medium with improved sensitivity over other available semiselective media for isolation of *X. campestris* pv. *campestris*. Differential colony morphology, rapid growth, and sharp hydrolysis zones on SM enable quick differentiation of *X. campestris* pv. *campestris* from other saprophytic bacteria, whereas recognition on TZC (5) was difficult when high populations of saprophytes were present. High plating efficiencies for most of the *X. campestris* pv. *campestris* strains tested on SM make this medium potentially valuable for diagnosis of black rot disease and for studies on survival of bacteria in the soil.

Based on the plating efficiency data, the SM medium is also useful for isolation of other xanthomonads. The medium also differentiates some *X. campestris* pathovars by color and colony morphology. Growth of some xanthomonads is not supported, however, and in other cases, starch hydrolysis is difficult to observe.

Starch was selected as the primary carbon source because it is hydrolyzed by most xanthomonads and many other organisms are reported to grow poorly or do not hydrolyze it at all (9). Methyl violet 2B and methyl green inhibit many Gram-positive bacteria (9) and the color accentuates the borders of starch hydrolysis zones, whereas brilliant cresyl blue (7) showed weaker zones. Glucose at a low concentration (0.1%) was incorporated to accelerate growth of *X. campestris* pv. *campestris* or to initiate growth of xanthomonads that hydrolyze starch slowly. Glutamic acid (7,10) and methionine (7,8,10) have been used to stimulate growth of xanthomonads. In our studies, starch hydrolysis zones appeared 1 day earlier when methionine

Table 5. Comparison of TZC, SX, and SM media in recovery of *Xanthomonas campestris* pv. *campestris* from infested soil

	Leaf/soil ratio (w/w)[a]					
	1:10		1:100		1:1000	
Medium	1	2	1	2	1	2
TZC	102/173[b]	?/TNTC[c]	34/74	44/93	12/29	11/31
SX	6/7	2/87	0/2	0/2	0	0
SM	61/62	99/160	55/59	59/64	9/10	15/15

[a] 10 g Unsterilized soil was infested with 1.0, 0.1, or 0.01 g naturally infected ground leaf material containing about 1×10^7 cfu/g as determined by repeated plating (six replicates) on TZC. Leaf-soil mixtures were extracted for 2 hr in 100 ml SDW and serially diluted; 0.1 ml of the 10^{-3} serial dilution was plated on media.

[b] Numbers = no. recognizable *Xanthomonas* colony-forming units (cfu)/total cfu. Average of two replicates.

[c] TNTC = too numerous to count; (?) = recognition of *X. Campestris* pv. *campestris* obscured by saprophytes.

rather than glutamic acid was used as a growth factor. A higher buffering capacity (0.025 M) than prescribed for other media (7,9) was used in SM to compensate for acid production during growth on NH_4Cl. Recognizable colonies appeared 1 day earlier within the pH range of 6.4–7.0 than at higher or lower pH.

Sodium chloride improved uniformity of colonies, which were raised, smooth, and convex. Tetrazolium chloride enhanced the differential pigmentation of *Xanthomonas* pathovars. Reduction of tetrazolium chloride appeared 1 day earlier (3 days) when trace elements were used. Cycloheximide was added at the rate used by Miller and Schroth (6); it inhibits yeasts, molds, and certain bacteria.

Although SM medium does not eliminate growth of all soil saprophytes, the latter can be differentiated from *X. campestris* pv. *campestris* by pigmentation and zones of starch hydrolysis. Addition of selective antibiotics such as kasugamycin (64 μg/ml) (B. N. Dhanvantari, *personal communication*) or pimaricin (50 μg/ml) (2) may provide further control of saprophytes.

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