Evaluation of a Selective Medium for Detecting *Pseudomonas syringae* pv. *papulans* and *P. syringae* pv. *syringae* in Apple Orchards

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ABSTRACT

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A selective, differential medium (PSM) devised for isolating *Pseudomonas syringae* pv. *papulans* (*Psp*) from plant tissues inhibited growth of most microorganisms and facilitated growth of some other *Pseudomonas* spp. including *P. syringae* pv. *syringae* (*Pss*). The medium proved superior to four other selective and differential media. *Psp* and *Pss* could be distinguished visually by differences in colony morphology. PSM was used to isolate *Psp* and *Pss* from dormant apple (*Malus pumila* 'Mutsu') buds, leaf scars, blossoms, fruits, and leaves and from five common orchard weeds.

Blister spot of apple (*Malus pumila*), which is caused by *Pseudomonas syringae* pv. *papulans* (Rose) Dhanvantari (6) (*Psp*) occurs primarily on the cultivar Mutsu in the northeastern United States and Canada (1,5). In New York state, 5–60% of the fruit in an orchard are typically infected, making them undesirable for the fresh market. Recently, we reported the periods during the growing season when fruit are susceptible to *Psp* (2). To improve the development of effective controls, certain aspects of the biology of the pathogen in orchards need to be investigated. This paper reports the development of a highly selective medium that can be used to study populations of *Psp* and *P. syringae* pv. *syringae* (*Pss*) on various plant tissues in apple orchards.

MATERIALS AND METHODS

Selective medium. A differential, selective agar medium was developed for the isolation of *Psp* and was found to support growth of some other *Pseudomonas* spp. including *Pss*. The medium (PSM) contains (g/L): sucrose, 50.0; proteose peptone #3 (Difco Laboratories, Detroit, MI 48232), 20.0; K_2HPO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 1.5; sodium taurocholate (Difco), 3.0; agar (Difco), 15.0; and 0.1 ml Tergitol 7 (Sigma Chemical Co., St. Louis, MO 63178). After autoclaving and cooling to 45 C, novobiocin, penicillin G, and cycloheximide (Sigma) were added aseptically to make final concentrations in the medium of 50, 60, and 100 mg/L, respectively. Other carbon and nitrogen sources including glycerol, D-glucose, mannitol, sorbitol, xylose, asparagine, and sodium tartrate and two selective growth-inhibiting compounds, dioctyl sodium sulphosuccinate and crystal violet, were tested as ingredients during the development of the selective medium. Final ingredients were selected for ability to inhibit contaminating bacteria from apple fruit and leaf washes without inhibiting growth of *Psp*.

Toxicity of selective media to *Pseudomonas* spp. PSM was compared with nutrient agar plus 5% sucrose (NAS) (23), King's Medium B (KB) (14), BCBRVB medium (21), and D-4 medium (13), or just to nutrient agar (NA) (Difco) for their relative toxicities to *Psp* and the other *Pseudomonas* spp. listed in Table 1.

Each isolate was grown on KB for 48 hr at 28 C and checked visually for purity. Cells from pure cultures were suspended in sterile distilled water (SDW) to make a final transmittance reading of 80%, as determined on a Bausch & Lomb Model 20 spectrophotometer (Bausch & Lomb, Rochester, NY 14625). Serial dilutions were made in water, and 0.1 ml of each dilution was spread in triplicate either on all four media or only on PSM and NA. Plates were incubated for 72 hr at 28 C before final colony counts were made. Platings of each isolate were repeated at least twice.

Identification of *Psp* on PSM. *Psp* on PSM was identified by the presence of a pale-blue fluorescence and flat, nonmucoid (levan negative [19]), cream-colored colonies. Other tests used to confirm

TABLE 1. Isolates of *Pseudomonas* spp. used to compare toxicities of selective media

Isolate	Strain	Host	Source[z]
P. syringae pv. *papulans*	CO-1	Mutsu apple fruit	1
papulans	0-1	Mutsu apple fruit	1
papulans	L-1	Mutsu apple fruit	1
papulans	S-1	Mutsu fruit wash	1
papulans	P-1	Mutsu fruit wash	1
papulans	C-3	Mutsu buds	1
syringae	Pss-C	Cherry canker	1
syringae	Pss-A	Apricot canker	1
syringae	B3A	Peach	2
syringae	B15	Almond leaf	2
syringae	GS28-1b	Prune canker	2
syringae	G55A	Bean	3
pisi	G28	Pea	3
tomato	G61A	Tomato	3
mors-prunorum	4	Tart cherry	4
mors-prunorum	5	Tart cherry	4
mors-prunorum	6	Tart cherry	4
atrofaciens	PA 14		5
aptata	PA 19		5
coronafaciens	PC 17		5
glycinea	PG 4		5
apii	PJ 1		5
lachrymans	PL 3		5
savastanoi	PS 29		5
tabaci	PT 11		5
P. angulata	NCPPB 1866		5
P. cepacia	PC 29		5
P. cichorii	PC 27		5
P. marginalis pv. *marginalis*	G42M	Parsnip	3
P. viridiflava	G21C	Parsnip	3
P. syringae pv. *phaseolicola*	G65A	Bean	3

[z] Sources of bacterial isolates: 1 = Authors'; 2 = H. English, Davis, CA; 3 = J. E. Hunter, Geneva, NY; 4 = A. L. Jones, East Lansing, MI; and 5 = R. S. Dickey, Ithaca, NY.

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the identity of *Psp* isolates were: presence of cytochrome oxidase (16), induction of a hypersensitive response on tobacco (15), liquefaction of gelatin (12), and hydrolysis of esculin (7). In addition, 50 isolates selected from PSM and identified as *Psp* according to the above criteria were verified by a testing scheme described previously (1) or by the following tests: presence of tyrosinase (19), casein proteolysis (7), production of acid from salicin (7), and utilization of tartrate and lactate (11,17). Isolates were also inoculated to Mutsu fruit in the orchard using the methods of Burr and Hurwitz (1). All tests for each isolate were repeated at least once.

The preceding testing schemes were also used to identify *Pss* isolates, which were often isolated from apple tissues.

Comparisons of selective media for isolation of *Psp* from leaves and soil. Media PSM, BCBRVB, D-4, NAS, and KB were compared for their effectiveness in detecting natural populations of *Psp* and in inhibiting growth of other bacteria from Mutsu leaf washes and orchard soils. Six 50-g samples of Mutsu leaves from five commercial and one experiment station orchard were collected during the 1980 growing season. All orchards had a previous history of blister spot. Leaves were randomly collected within each orchard and stored in Ziploc plastic bags (Dow Chemical Co., Indianapolis, IN 46268).

Ten small samples of soil (5–10 cm in depth) from each of three commercial Mutsu orchards were collected within the drip line of trees that bore naturally infected fruits. Each composite sample of ~2 kg was placed in a plastic bag. Both soil and leaf samples were transported to the laboratory in an ice chest and processed within 2 hr.

Soil samples were thoroughly mixed and 10-g subsamples were removed and placed in flasks for washing and diluting. Soil and leaf samples were washed by continuous shaking in 1 L SDW on a wrist-action shaker (Burrell Corp., Pittsburgh, PA 15238) for 2 hr. Serial water dilutions were made and 0.1-ml aliquots were plated in triplicate on all five media. Plates were incubated at 28 C for 72 hr before colonies of *Psp* and other bacteria were counted. Final counts were made at 10 days. All other tissue samples were processed as above unless otherwise specified.

Isolations from other plant tissues. A total of 133 samples of Mutsu blossoms, leaves, and fruits were collected during 1978, 1979, and 1980 from 10 orchards located throughout New York state. One hundred blossoms or leaves, and 100, 50, or 25 fruits were randomly collected for each sample. Samples were washed as described previously and dilutions were plated on PSM.

In addition, isolations were attempted from overwintering, infected Mutsu fruit, dormant buds, and leaf scars and from leaves of various orchard weeds. Naturally infected Mutsu apples were placed under trees in an orchard at Geneva in November 1978. Each month through the following April, several lesions were macerated in SDW and loopfuls of the suspension were streaked onto PSM.

Dormant Mutsu buds were examined in February 1980 as potential sites of overwintering inoculum. Fifty to 100 buds from each of five orchards were sampled individually or in groups of 10 by washing for 2 hr in 10 ml of SDW, or by homogenizing them in a Tissuemizer (Model SDT-182 EN, Tekmar Co., Cincinnati, OH 45222). Five hundred leaf scars from the same orchards were homogenized in groups of 10 in 10 ml of SDW. Subsequently, serial dilutions were made, and 0.1-ml aliquots of the suspensions were plated in triplicate on PSM.

Leaves of orchard weeds were sampled periodically for *Psp* during the 1979 and 1980 growing seasons. Fifty-gram samples from *Taraxacum officinale* Weber (dandelion), *Agropyron repens* L. (quackgrass), *Euphorbia esula* L. (leafy spurge), *Plantago major* L. (common plantain), *Amaranthus hybridus* L. (green amaranth), *Trifolium* sp. (clover), and *Malva neglecta* L. (common mallow) were randomly selected, washed in 1 L SDW, and plated on PSM. All samples were placed in Ziploc bags, transported to the laboratory in an ice chest, and processed within 2 hr.

RESULTS

Toxicity of PSM to *Pseudomonas* spp. PSM was relatively nontoxic to *Psp*, *Pss* and to some other *Pseudomonas* spp. (Tables 2 and 3). NAS and KB were generally less toxic, and D-4 was most toxic to selected isolates. *Psp* colonies of ~2-mm diameter developed on PSM and BCBRVP after 72 hr of incubation at 28 C. Generally, 24–48 hr was sufficient for equal sized colonies to develop on NAS and KB. Final colony counts usually were made after 72 hr, with the exception of D-4 medium, which required 72 hr for *Pss* colonies to develop and up to 10 days before *Psp* colonies could be detected.

Identification of *Psp* and *Pss*. *Psp* could be identified by colony morphology (levan negative) (Fig. 1) and pale blue fluorescence of colonies on PSM, induction of a hypersensitive response on tobacco, hydrolysis of esculin, inability to liquify gelatin, and absence of cytochrome oxidase. Fifty selected isolates of *Psp* reacted negatively for the following tests: acid from salicin, casein proteolysis, utilization of tartrate, and production of pectolytic enzymes at pH 5.0, 7.0, and 8.0. Isolates were positive for lactate utilization and a variable response was obtained for the presence of tyrosinase. Except for the lack of acid production from salicin, these results agree with those reported by Dhanvantari (6).

Typical blister spot symptoms developed within 2 wk on all fruits inoculated with the 50 *Psp* isolates in the orchard. Symptom development was identical to that occurring from natural infection.

A major advantage of PSM is that *Psp* could be distinguished from *Pss* by colony morphology. *Pss* colonies were rounded and mucoid (levan positive) (Fig. 1) and had a more intense fluorescence on PSM. Oxidase-positive Pseudomonads also grew on PSM, but these usually had levan-positive type colonies that were larger than *Pss*.

Detection of natural populations of *Psp* on selective media. PSM was highly selective and differential for the isolation of *Psp* from apple leaves and eliminated growth of over 90% of other bacteria (Table 4). BCBRVP also was selective, but not differential for *Psp*, and it allowed more contaminating bacteria to grow. Medium D-4 eliminated more contaminating bacteria than other media. However, it was also most toxic to *Psp* (Table 4).

No *Psp*, *Pss* or other oxidase-negative pseudomonads were

TABLE 2. Relative plating efficiencies of PSM and other media for *Pseudomonas syringae* pv. *papulans* (*Psp*) and *P. syringae* pv. *syringae* (*Pss*)

	Colonies detected (avg no. per 0.1 ml)[y]											
	Psp isolates						*Pss* isolates					
Medium[z]	C-3	S-1	L-1	O-1	P-1	Co-1	Apricot	Cherry	B-15	RMs-108	GS28-1b	RMs-118
NAS	35.0 ab	19.7 b	39.7 ab	47.7 a	54.0 a	33.7 b	13.0 a	48.3 a	21.3 ab	29.3 a	28.7 a	26.0 a
KB	32.7 b	33.7 a	40.0 a	40.3 b	48.7 a	41.7 a	11.3 a	46.0 ab	18.3 b	24.0 a	26.3 a	22.3 a
PSM	24.3 c	30.3 a	43.0 a	40.0 b	53.0 a	36.3 ab	10.0 a	21.0 c	21.7 ab	28.0 a	24.7 a	17.3 ab
BCBRVB	39.3 a	34.0 a	30.7 b	43.0 b	50.3 a	33.7 b	9.7 a	33.3 bc	27.0 a	26.7 a	20.7 a	24.7 a
D-4	0.0 d	3.3 c	0.0 c	1.0 c	0.0 b	0.0 c	1.0 b	8.0 d	18.7 b	12.7 b	6.7 b	10.0 b

[y]Serial water dilutions of an 80% transmittance suspension of cells of each isolate were plated (0.1 ml) in triplicate on each medium. Values for each isolate were analyzed using the Waller-Duncan BSD Rule. Values within each column, followed by the same letter are not significantly different ($P = 0.01$). The experiment was repeated at least twice for each isolate.

[z]NAS = Nutrient agar plus 5% sucrose, KB = King's Medium B (14), PSM = Authors' medium, BCBRVB = medium of Sands and Scharen (21), and D-4 = medium of Kado and Heskett (13).

detected in orchard soils. Total bacteria were counted from each of the soil samples from the three orchards. In all three cases, medium D-4 yielded the fewest bacteria (1.5×10^3, 1.0×10^3 and 6.0×10^3 colony-forming units [cfu] per gram of soil) followed by PSM (1.8×10^4, 2.3×10^3, 9.7×10^3), BCBRVP (4.7×10^4, 5.9×10^4, 4.0×10^4) NAS (5.4×10^4, 7.1×10^4, 7.4×10^4) and KB (1.0×10^5, 4.7×10^4, 1.2×10^5). These populations show the average number of bacteria from three replicates, recovered from each soil sample respectively. PSM eliminated 82, 95, and 92% of the soil bacteria from the three samples as compared to KB.

Isolations from plant tissues. Populations of *Psp* detected in several commercial orchards in the major fruit-growing areas of central and western New York are given in Table 5. The bacterium frequently was isolated from Mutsu blossoms, leaves, and fruits. *Pss* also was isolated from a high percentage of blossom, leaf, and fruit samples with populations ranging as high as 7.7×10^1, 6.9×10^2 and 3.1×10^2 cfu per blossom, leaf or fruit, respectively. These data show the widespread occurrence of these two *Pseudomonas* pathovars in orchards throughout the growing season.

Psp was isolated from lesions on Mutsu fruits collected from the orchard floor from November through April. Although overwintering fruits decayed rapidly during the spring, enough tissue remained intact to harbor inoculum until new green leaf tissues developed on trees.

The bacterium was detected in 40% of the dormant buds in one orchard. From four other orchards, where various numbers of 10-bud samples were assayed, 6 of 10, 7 of 8, 4 of 5, and 4 of 10 yielded *Psp*. These data indicate that high percentages of dormant apple buds can be infested with *Psp* and that they serve as a primary site for overwintering of inoculum. However, only two isolates from 500 buds were obtained from surface washes, when buds were not ground. *Psp* was recovered from only one of 50, 10-leaf scar samples. In addition to *Psp*, *Pss* frequently was isolated from ground bud samples.

PSM also was used to recover *Psp* and *Pss* from leaves of *T. officinale*, *A. repens*, *E. esula*, *Trifolium* sp., and *M. neglecta*. Neither bacterium was detected from leaves of *P. major* or *A. hybridus*.

TABLE 3. Plating efficiency of *Pseudomonas* spp. on PSM medium

Isolate	Colonies detected[x] (avg no. per 0.1 ml) NA[y]	PSM	Plating efficiency (%)[z]
P. syringae pv. *mors-prunorum* (4)	34	28	82
mors-prunorum (5)	31	0	0
mors-prunorum (6)	18	0	0
syringae (bean)	35	41	117
phaseolicola	34	32	94
pisi	32	32	100
tomato	36	32	89
atrofaciens	23	2	9
aptata	31	13	42
coronafaciens	28	0	0
glycinea	28	19	68
apii	29	6	21
lachrymans	32	11	34
savastanoi	35	23	66
tabaci	27	0	0
P. marginalis pv. *marginalis*	32	36	113
P. viridiflava	28	28	100
P. angulata	30	26	87
P. cepacia	28	0	0
P. cichorii	34	34	100

[x] Serial water dilutions of an 80% transmittance suspension of cells of each isolate were plated (0.1 ml) in triplicate on each medium. Counts represent the average no. of colonies per plate for two experiments, each including three replica plate counts per isolate. The experiment was repeated at least twice for each isolate.
[y] Abbreviations: NA = Nutrient agar (Difco) and PSM = Authors' medium.
[z] Plating efficiency = (PSM/NA) ×100.

DISCUSSION

PSM is superior to other selective and differential media (13,14, 21,23) for isolating natural populations of *Psp*. Not only is the medium relatively nontoxic and eliminates over 90% of contaminant bacteria from leaves, it is also differential in that *Psp* colonies are easily distinguished from *Pss*. The ability to distinguish

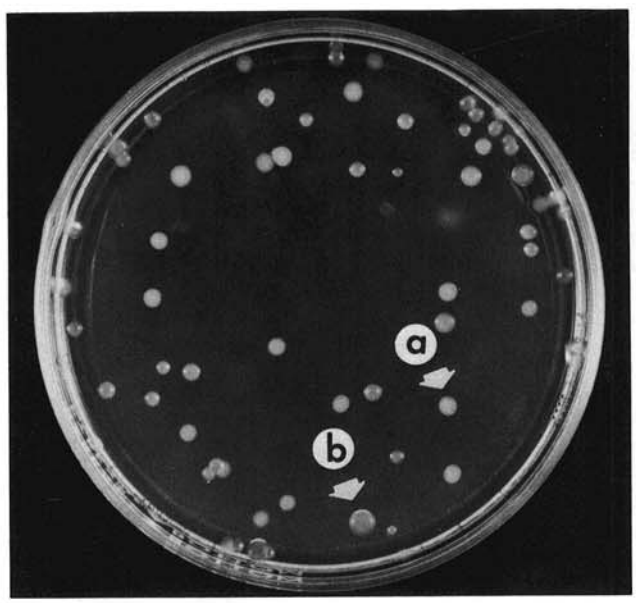

Fig. 1. Typical flat, cream-colored, nonmucoid colonies of **a,** *Pseudomonas syringae* pv. *papulans* and **b,** raised, slimy, translucent colonies of *P. syringae* pv. *syringae* isolated on PSM medium from wash water after cleaning a Mutsu apple fruit.

TABLE 4. Recovery of *Pseudomonas syringae* pv. *papulans* (*Psp*) and other bacteria from Mutsu apple leaf washes on various selective and differential media

Media[z]	Bacterial populations (cells × 10^4 per milliliter of wash water)[y] Orchard A *Psp*	Orchard A Other	Orchard B *Psp*	Orchard B Other	Orchard C *Psp*	Orchard C Other	Orchard D *Psp*	Orchard D Other	Orchard E *Psp*	Orchard E Other	Orchard F *Psp*	Orchard F Other
NAS	4.70	11.00	0.00	5.00	0.00	9.00	0.00	8.80	0.00	7.30	0.00	9.00
KB	3.70	7.30	0.00	2.20	0.00	7.30	0.00	8.70	0.00	7.00	0.00	47.00
PSM	5.00	0.00	0.07	0.53	0.02	0.10	0.03	0.93	0.03	1.40	0.10	1.90
BCBRVB	3.00	2.70	0.03	1.90	0.03	3.40	0.00	3.50	0.03	2.20	0.07	4.40
D-4	0.77	0.07	0.00	0.43	0.01	0.04	0.00	0.03	0.00	0.01	0.00	1.10

[y] Fifty grams of leaves were collected from each orchard and washed in 1 L of distilled water for 2 hr; 0.1 ml of serial water dilutions were plated in triplicate on each medium. Plates were incubated at 28 C for 3–10 days.
[z] Abbreviations: NAS = nutrient agar + 5% sucrose, KB = King's Medium B (14), PSM = Authors' medium, BCBRVB = medium of Sands and Scharen (21), D-4 = medium of Kado and Heskett (13).

TABLE 5. Populations of *Pseudomonas syringae* pv. *papulans* (*Psp*) recovered from Mutsu blossoms, leaves and fruits[v]

Orchard	Freq. of recovery from			Populations associated with[w]		
	Blossom	Leaf	Fruit	Blossom	Leaf	Fruit
Station	1/2[x]	3/4	8/12	2.3×10^0	3.2×10^2	1.5×10^3
Cohn	1/4	4/4	25/34	7.7×10^1	5.4×10^3	1.4×10^5
Red Jacket	3/5	12/12	11/12	8.8×10^3	5.4×10^4	1.5×10^4
Brown	3/3	NS[y]	2/2	7.0×10^2	–	7.4×10^3
Smith	NS	8/8	2/2	–	4.0×10^2	3.3×10^3
VanAcker	NS	2/8	2/6	–	5.2×10^1	4.0×10^2
Oakes	NS	1/1	9/10	–	2.3×10^0	2.7×10^3
Sodoma	NS	NS	2/2	–	–	3.5×10^3
Pearson	NS	NS	1/1	–	–	1.6×10^2
Kirby	NS	NS	0/1	–	–	ND[z]

[v] One hundred blossoms, leaves, and 100, 50, or 25 fruits were randomly collected periodically from 24 May to 7 September, 1978, 25 April to 24 August, 1979, and from 12 May to 8 October, 1980. Samples were washed in 1 L sterile, distilled water and 0.1 ml serial water dilutions were plated in triplicate on PSM medium.

[w] The highest population of *Psp* detected per blossom, leaf, or fruit is given.

[x] Fractions indicate the number of samples from which *Psp* was recovered divided by the total number of samples.

[y] NS = not sampled.

[z] ND = none detected.

between these pathovars on PSM is necessary when studying the epidemiology of blister spot, since we have recently shown that *Pss* does not cause the disease (2). Since other *Pseudomonas* spp. including *Pss* grow well on PSM and the medium is quite toxic to the majority of soil bacteria, the medium may be useful in studying the biology of these pathogens in the field. Previously, little data concerning the biology of *Psp* in apple orchards were available. Our investigations indicate that the bacterium may overwinter in apple buds and in infected fruit on the orchard floor. Considering the relatively few buds per orchard that were sampled and the high frequency with which *Psp* was recovered, it is likely that buds constitute a major site for overwintering inoculum. It also appears that the bacterium survives within the buds and is protected by bud scales, since recovery was greatly enhanced by homogenizing them prior to plating. Further tests have shown that shortly after growth of new leaf tissue begins in the spring, *Psp* can be detected by washing and plating dilutions on PSM (*unpublished*).

This report shows that *Psp* and *Pss* survive as epiphytes (18) on apple blossoms, leaves and fruits, and on some orchard weeds. Since differential washing times and techniques were not utilized in this study, it is possible that some of the reported bacterial populations may not be epiphytes. Experiments have been conducted, however, to demonstrate that a 2-hr wash time is optimum for maximum recovery of *Psp* from apple leaves (Burr and Katz, *unpublished*). *Psp and Pss* were detected in apple orchards throughout the growing season. Populations probably will vary with location of the tissues in the tree canopy, tissue maturity, and environmental conditions prior to sampling. Such factors are reported to affect *Pss* populations on sweet cherry (3). Future control strategies for blister spot may involve monitoring epiphytic populations of *Psp*, especially during periods when fruit are highly susceptible (2).

The recovery of *Pss* from weed leaves was not surprising since many reports have demonstrated this relationship (4,8,17). It is interesting, however, that the bacterium was frequently detected from apple tissues where it rarely incites a disease. *Pss*, for instance, does not cause blister spot (2) and only rarely has been reported to cause blossom infections (9,10) or cankers (22) on apple. Only one report has indicated that such a problem may occur rarely in New York (20). The possibility that *Pss* and *Psp* may be involved with frost injury and bud mortality needs further investigation.

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