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NOTES

Selective Medium for Isolation of *Xanthomonas maltophilia* from Soil and Rhizosphere Environments†

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A selective medium (XMSM) was developed for isolation of *Xanthomonas maltophilia* from bulk soil and plant rhizosphere environments. The XMSM basal medium contained maltose, tryptone, bromthymol blue, and agar. Antibiotics added to select for *X. maltophilia* were cycloheximide, nystatin, cephalexin, bacitracin, penicillin G, novobiocin, neomycin sulfate, and tobramycin. A comparison was made between XMSM and 1/10-strength tryptic soy broth agar for recovery of *X. maltophilia* from sterile and nonsterile soil infested with known *X. maltophilia* isolates. A recovery rate of 97% or greater for XMSM was demonstrated. XMSM was used to isolate *X. maltophilia* from a variety of soil and rhizosphere environments.

Xanthomonas maltophilia, formerly *Pseudomonas maltophilia* (13), is a common cause of opportunistic infections in humans (3). Recently, it was shown that it often composes a portion of the bacterial microflora of soils and plant rhizospheres (1, 5). In addition, *X. maltophilia* has been suggested to have properties useful in the biological control of soilborne plant pathogens (2, 7, 8). Although *X. maltophilia* does grow on media selective for pseudomonads (e.g., King medium B), there is no selective medium which separates this saprophytic xanthomonad from pseudomonads. The development of a selective medium for *X. maltophilia* and preliminary results from the use of this medium are presented.

Since a taxonomically significant characteristic of *X. maltophilia* is its ability to produce acid aerobically from maltose, the maltose medium of Hugh and Ryschenkow (4), modified by the substitution of tryptone (Difco Laboratories, Detroit, Mich.) for Casitone (Difco), was used as an initial basal medium. Other compounds tested for differentiation of *X. maltophilia* were Tween 80 (10 ml/liter; Fisher Scientific Co., Pittsburgh, Pa.) and potassium bromide (10 g/liter; Sigma Chemical Co., St. Louis, Mo.). Tween 80 is used to detect lipolytic activity (11), a common characteristic of *X. maltophilia* strains. Bromine enhances the yellow pigment of phytopathogenic xanthomonads (9).

Compounds tested for antifungal activity were benomyl, cycloheximide, and nystatin. Bacitracin, chloramphenicol, penicillin G (sodium salt), and vancomycin were tested at 10 and 25 μg/ml for inhibition of gram-positive bacteria. The following chemicals and antibiotics were tested for inhibition of gram-negative bacteria, excluding *X. maltophilia* (in micrograms per milliliter): gentamicin sulfate and tobramycin (1 and 5); ampicillin, colistin methanesulfonate, kanamycin, lincomycin, methyl green, nalidixic acid, neomycin sulfate, novobiocin, polymyxin B sulfate, and tetracycline (5 and 25); erythromycin, 5-fluoruracil, and kasugamycin (10 and 25); cephalexin (50); thionin and lead acetate (10); and dodecyltrimethylammonium bromide (100). Many of the compounds were examined on the basis of previous research with *X. maltophilia* and *Xanthomonas campestris* (9, 13, 14). All antibiotics were obtained from Sigma. Benomyl was obtained from E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. Other chemicals were obtained from Sigma or Fisher Scientific.

Bacteria representative of soil and rhizosphere environments (Table 1) were first screened for ability to produce acid aerobically from maltose. Acid was consistently produced by all *X. maltophilia* isolates, *Erwinia carotovora* subsp. *carotovora*, *X. campestris* pv. *sojense*, *Curtobacterium flaccumfaciens* pv. *betae*, and *Flavobacterium meningosepticum*. The acid reactions for the last three organisms were usually weak compared with that of *X. maltophilia*. Inconsistent acid production was obtained with a few *Bacillus* isolates and one *P. fluorescens* isolate.

However, these bacteria could be effectively inhibited with antibiotics such that *X. maltophilia* could be selectively isolated. Cephalexin inhibited *E. carotovora* subsp. *carotovora*. As a combination, bacitracin and penicillin G controlled growth of the gram-positive bacteria. Novobiocin effectively inhibited *F. meningosepticum* and the *X. campestris* pathovars. Neomycin sulfate inhibited *Pseudomonas* species and *X. campestris* pathovars, whereas a low level of tobramycin (1 μg/ml) inhibited *Pseudomonas* species only. Although the levels of neomycin sulfate and tobramycin used alone did not completely suppress the growth of all *Pseudomonas* species, together these antibiotics were effective. The same was true for neomycin sulfate and novobiocin, which together completely inhibited all *X. campestris* pathovars. The other antibiotics tested either inhibited the growth or acid production of *X. maltophilia* or did not effectively inhibit the other gram-negative bacteria.

Since the fungicide benomyl repressed the acidic reaction of *X. maltophilia*, cycloheximide and nystatin were used as fungal inhibitors. The addition of Tween 80 also interfered

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TABLE 1. Bacteria used in developing the XMSM selective medium for *X. maltophila*

Bacterium	Source	No. of isolates
Clavibacter michiganense subsp. *nebraskense* (298)	M. Davis	1
Curtobacterium flaccumfaciens pv. *betae* (NCPPB 374)	M. Davis	1
Pseudomonas marginalis (ATCC 10844)	M. Davis	1
Pseudomonas alcaligenes (ATCC 14909)	M. Davis	1
Pseudomonas fluorescens (28, 165, 199)	M. E. Juhnke	3
Erwinia carotovora subsp. *carotovora* (ATCC 15713)	M. Davis	1
Flavobacterium meningosepticum (DCC 3405)	M. Sasser	1
Streptomyces griseus (D-185)	M. E. Juhnke	1
Bacillus subtilis (D-39, D-60, 1001, ATCC 6633)	M. E. Juhnke	4
Bacillus pumilus (D-188)	M. E. Juhnke	1
Bacillus spp. (D-175, D-221)	M. E. Juhnke	2
Xanthomonas albilineans (XACP-4)	M. Davis	1
Xanthomonas campestris		
pv. *translucens* (ATCC 10731, DCS87-80X)	L. Claflin, D. Sands	2
pv. *malvacearum* (race 4, strain H)	D. Gabriel	1
pv. *phaseoli* (Wallace Co.)	L. Claflin	1
pv. *sojense* (Horticultural Farm)	L. Claflin	1
pv. *hederae* (NZ 1661)	L. Claflin	1
pv. *alfalfae* (LY 4401)	L. Claflin	1
Xanthomonas maltophilia (59, 88, 1000)	M. E. Juhnke	3
Xanthomonas maltophila (ATCC 53199)	H. Hoitink	1

with acid production by *X. maltophilia*, and potassium bromide did not enhance the yellow pigmentation associated with the *X. maltophilia* isolates tested. Therefore, these two compounds were not incorporated into the selective medium.

The final selective medium (XMSM) contained (per liter): maltose (10 g) (Fisher Scientific), tryptone (5 g) (Difco), bromthymol blue (4 ml of 2% aqueous solution) (Sigma), and Bacto-Agar (15 g) (Difco). After the medium was autoclaved and cooled to 50°C, pH was adjusted to 7.1 with 1 N NaOH, which produced a green coloration of the medium. The following antibiotics were then added (in micrograms per milliliter): cycloheximide (100), nystatin (50), cephalexin (50), bacitracin (25), penicillin G (25), novobiocin (10), neomycin sulfate (30), and tobramycin (1).

The *X. maltophilia* isolates used in the initial screening of XMSM produced orange colonies with a yellow halo in the medium because of acid production from maltose. This acidic reaction was best observed after 2 days of growth and often faded after 4 to 6 days of growth.

Five *X. maltophilia* isolates (one from Ohio, two from Montana, and two from Florida) were used to determine the isolation efficiency of XMSM for *X. maltophilia* from sterile soil. These isolates had been obtained from bulk soil or plant rhizosphere environments. Isolates were grown on tryptic soy broth agar (TSBA) plates (30 g of tryptic soy broth and 15 g of agar per liter). For each isolate, cells from one TSBA plate were suspended in 10 ml of sterile phosphate buffer (pH 6.8; 8.5 g of NaCl, 11.4 g of $K_2HPO_4 \cdot 3H_2O$, and 6.8 g of KH_2PO_4 per liter). The cell suspension ($\log_{10}$ 9.7 to $\log_{10}$ 9.9 CFU/ml) was then mixed with 100 g of sterilized Bozeman silt loam soil; the control was 10 ml of sterile phosphate buffer added to soil. Inoculated soils were covered and incubated at room temperature. After 2 days of incubation, 1 g of soil was removed, placed in 99 ml of sterile phosphate buffer, and shaken on an orbital shaker for 10 min at 180 rpm and 28°C. Serial dilutions were plated in duplicate on XMSM and 1/10-strength TSBA supplemented with cycloheximide (100 μg/ml) and nystatin (50 μg/ml). Plates were incubated at 28°C, and the colonies were counted after 2 and 4 days of growth. Recovery of *X. maltophilia* from soil with XMSM, compared with recovery with antibiotic-supplemented 1/10 TSBA, ranged from 97 to 100% for the five isolates (Table 2). This experiment was repeated by using sterilized and unsterilized Bozeman silt loam soil and *X. maltophilia* isolates 88 (Montana) and ATCC 53199 (Ohio). The recovery values were, again, 97% or greater for both soil treatments and both isolates.

TABLE 2. Recovery of *X. maltophilia* isolates from sterile soil infested with the isolates by using a selective medium (XMSM) and a general plate count agar medium

X. maltophilia isolate	Source of isolate	$\log_{10}$ CFU/g of soil[a]	
		XMSM	$\frac{1}{10}$ TSBA[b]
ATCC 53199	Ohio	7.6	7.8
Xm-1	Montana	7.4	7.6
88	Montana	7.7	7.8
Xm-25	Florida	7.4	7.4
Xm-29	Florida	7.7	7.8
Control		0[d]	0[d]

[a] Values are averages of two replications.
[b] Supplemented with the antifungal antibiotics used in XMSM.
[c] No bacterial isolates were added to the sterile soil.
[d] No bacteria were observed.

To determine the isolation efficacy of XMSM for recovery of *X. maltophilia*, soil or plant root samples were collected from 1 location in Montana and 19 locations in southeastern Florida. Soil and root samples were obtained from golf courses, home lawns, a grass pasture, and heliconia plants grown in greenhouse container media. Soil samples were obtained from a cereal grain field, a sugarcane field, palm groves, and an orange grove. For soil samples, 1 g of soil was placed in 99 ml of sterile phosphate buffer and shaken as described above. Serial dilutions were plated on XMSM and, for some samples, on antibiotic-supplemented 1/10 TSBA. For root samples, approximately 1 g (wet weight) of roots with closely adhering soil was placed in 100 ml of sterile phosphate buffer and then processed as were the soil samples. The proportion of *X. maltophilia* to other bacteria growing on XMSM ranged from 0 to 100% (Table 3). The proportion of *X. maltophilia* growing on XMSM to total bacteria growing on 1/10 TSBA was determined for 10 samples and was <1% for all samples.

Colonies on XMSM representative of morphological types present were selected, purified, and stored on 1/10 TSBA slants at 4°C. Isolates were identified by using the scheme outlined in *Bergey's Manual of Systematic Bacteriology* (6), supplemented with information from Swings et al. (13), Palleroni (10), and Stolp and Gadkari (12). The following tests were used for identification: Gram staining, oxidase reaction, catalase reaction, anaerobic growth, starch hydrolysis, Tween 80 hydrolysis, production of fluorescent pigments, flagellum staining, production of acid aerobically

TABLE 3. Total number of bacteria and number of *X. maltophilia* from bulk and rhizosphere soil samples isolated with selective medium XMSM

Location code	Plant host	Soil type	CFU, 10^1		% *X. maltophilia*
			X. maltophilia	Total bacteria	
1	Bermuda grass	Rhizosphere	1	37	3
	Bermuda grass	Bulk	0	86	0
2	Bermuda grass	Rhizosphere	80	80	100
3	Bermuda grass	Rhizosphere	5	26	19
	Bermuda grass	Bulk	35	197	18
4	Bermuda grass	Rhizosphere	1,490	2,900	51
	Bermuda grass	Bulk	4	4	100
5	Bermuda grass	Rhizosphere	180	1,300	14
	Bermuda grass	Bulk	4	19	21
6	St. Augustine grass	Rhizosphere	300	600	50
7	St. Augustine grass	Rhizosphere	52	125	42
	St. Augustine grass	Bulk	2	2	100
8	St. Augustine grass	Rhizosphere	410	1,080	38
	St. Augustine grass	Bulk	110	130	85
9	St. Augustine grass	Rhizosphere	100	1,730	6
19	St. Augustine grass	Bulk	10	10	100
10	Unknown pasture grasses	Rhizosphere	100	5,700	2
	Unknown pasture grasses	Bulk	400	590	68
11	Bahai grass	Rhizosphere	3	7	43
	Bahai grass	Bulk	1	4	25
12	Bahai grass	Rhizosphere	430	1,000	43
	Bahai grass	Bulk	2	28	7
13	Heliconia	Rhizosphere	2	480	<1
	Heliconia	Bulk	3	108	3
14	Sugarcane	Bulk	9	43	21
15	Palm grove	Bulk	0	43	0
16	Palm grove	Bulk	10	39	26
17	Palm grove	Bulk	12	93	13
18	Orange grove	Bulk	0	53	0
20	Wheat or barley	Bulk	42	111	38

from maltose, and growth in the absence of growth factors such as methionine. Of 69 bacterial isolates examined, 44 were *X. maltophilia*.

In general, the *X. maltophilia* isolates had circular (5-mm diameter), entire, convex, orange-yellow colonies with or without an acidic (yellow) halo. The colony color was either completely orange or dark yellow or had a "fried-egg" appearance, with an orange center surrounded by a yellow collar. Typical colony appearance was best observed after 2 days of growth. On 1/10 TSBA, these isolates were fast growing and yellow. All 44 *X. maltophilia* isolates were aerobic, oxidase-negative, catalase-positive, gram-negative rods with multitrichous polar flagella. The isolates did not fluoresce on King medium B, hydrolyze starch, or grow in the absence of growth factors. All isolates produced acid aerobically from maltose; only one did not produce lipase.

The 25 isolates which were not confirmed as *X. maltophilia* were either small (less than 2 mm in diameter) and yellow or small and translucent after 2 days of growth on XMSM. On 1/10 TSBA, these isolates were either slow growing and yellow or yellow with copious amounts of slime produced. Although these non-*X. maltophilia* isolates were gram-negative rods also, they were usually oxidase positive, did not produce acid aerobically from maltose, or had only one polar flagellum.

The selective medium XMSM was used successfully in the isolation of *X. maltophilia* from various plant and soil environments. All strains produced typical orange or yellow colonies which could be differentiated from the limited number of gram-negative bacterial contaminants that also grew on XMSM. Selective media are invaluable in the study of specific bacterial groups. We believe that XMSM will provide another tool for researchers studying soil and plant microbial ecology.

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