Selective Media for Isolation of Agrobacterium, Corynebacterium, Erwinia, Pseudomonas, and Xanthomonas

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ABSTRACT

Five selective plating media (designated as D-series media) were developed for plant-pathogenic bacteria in the genera *Agrobacterium, Corynebacterium* (including species pathogenic for animals and man), *Erwinia, Pseudomonas,* and *Xanthomonas*. The active constituents of these media were lithium chloride, sodium dodecyl sulfate, polymyxin, and glycine, all of which are known to affect the permeability of bacterial membranes. These selective media were designed using one or a combination of these compounds to permit the growth of bacteria of one genus and restrict the growth of all others. Using inoculum containing a mixture of different bacteria, the efficiencies of these media are as follows: Plating efficiencies ([colony numbers on selective medium: colony numbers on nonselective medium] $\times 10^2$) were 90.3% for *A. tumefaciens* on medium D1; 27.1% for *C. michiganense* on medium D2; 77.7% for *E. amylovora* on medium D3; 6.5% for *P. syringae* on medium D4; and 78.4% for *X. campestris* on medium D5. Recovery efficiencies ([colony numbers from soil on selective medium: colony numbers without soil on the same selective medium] $\times 10^2$) of the respective bacteria from soil artificially inoculated with a mixture of bacteria were 48.1% on D1, 79.8% on D2, 57.0% on D3, 26.9% on D4, and 79.9% on D5. Recovery of the pathogenic bacteria from diseased tissues was facilitated by these media. Phytopathology 60:969-976.

Standard procedures for isolating plant-pathogenic bacteria from diseased material and soil are facilitated by use of selective and diagnostic media. Media commonly employed, however, are nutrient agar and media containing tryptone, peptone, beef extract, etc., supplemented with one or more sugars. These media were designed initially for bacteria whose ecological niche involves animals, and are used for isolating and identifying bacteria primarily of public health and medical interests. It has been difficult to design media for selecting plant-pathogenic bacteria. Some media have been designed for bacteria that develop distinctive colony characteristics so that the plant pathogens may be distinguished in a population of saprophytes. Other media have been more or less selective for certain bacteria, i.e., *Agrobacterium tumefaciens* (22, 24, 28, 34); *Corynebacterium* species (4, 7, 17, 19, 32); *Erwinia carotovora* (20, 30); *E. stewartii* (10); *E. rubrifaciens* (26); and *Pseudomonas mors-prunorum* (2). A medium containing tetrazolium salts used for differentiating mutants of *Escherichia coli* (15) has been modified and used to differentiate pathogenic isolates of *P. solanacearum* (12), and *E. carotovora* from *E. atroseptica* and *E. aroideae* (16). *Pseudomonas* species were isolated with a medium containing taurocholate or crystal violet as the selective agent (3, 36).

Few media have been designed for *Xanthomonas*; pathogenic cultures of *Xanthomonas* have been maintained on a medium containing commercial carrot juice in glucose-yeast extract-calcium carbonate agar (27), and also on the same medium without carrot juice.

The selectivity and differentiating abilities of most media are based on preventing or retarding the growth of cells by using inhibitors of metabolic pathways (e.g., heavy metals, crystal violet) and antibiotics that suppress protein and nucleic acid synthesis (e.g., streptomycin, neomycin, novobiocin, actinomycin D).

Our media (designated as D-series media) were designed principally on the basis of altering the surface components and membrane of the bacterial cell as proposed earlier (11). Chosen as constituents, therefore, were compounds such as sodium dodecyl sulfate, lithium chloride, glycine, and polymyxin. The advantages of using such compounds are that they eliminate the necessity of using inhibitors of protein and nucleic acid synthesis and allow ordinary constituents in the medium to control growth. Also, there are other advantages of our media. For instance, the primary purpose of diagnostic work is the isolation of the pathogen, but efficient use of labor, material, and time becomes increasingly important as the number of diseased specimens in the laboratory increases. The use of medium that is specifically designed to isolate species of a given genus greatly increases the efficiency and provides a minimum of further investigation. Furthermore, rarely has there been data on the plating and recovery efficiencies of useful media. We have felt a need for such media as well as a general medium designed specifically for plant-pathogenic bacteria. Thus, the D-series media were developed avoiding strictly empirical methods by employing multiple titrations of the active constituents of each medium, by replica plating, and based on selectively altering membranes of bacteria by the above compounds. The ingredients and properties of selective plating media are presented.

Materials and Methods.—The following constituents were used: Yeast extract and Bactoagar (Difco Laboratories); casein enzymatic hydrolysate (California Biochemical Corporation); Polymyxin sulfate B and cellobiose (Nutritional Biochemicals Corporation); bromthymol blue and acid fuchsin (Allied Chemical Corporation); Tris [tris (hydroxymethyl) amino methane] and glycine (Sigma Biochemical Corporation); and sodium dodecyl sulfate (reagent-grade,

95% pure, recrystallized from ethanol, Matheson, Coleman and Bell Company). All other chemicals were of analytical reagent grade (Mallinkrodt Chemical Works).

Cultures.—Sources of cultures are listed in Table 1.

Replica plating.—Template replication was performed by transferring individual colonies with sterile toothpicks from plate to plate using grids containing 120 quadrants as guides. This procedure reduces the possibility of carrying over excessive quantities of cells and nutrients during clonal transfer (1), and prevents the papillae colonies (14, 25, 31, 36) commonly encountered with streaking techniques.

Media and buffers.—Medium 523 used as our general medium contains, per liter: 10 g sucrose, 8 g casein hydrolysate, 4 g yeast extract, 2 g K_2HPO_4 (or 2.4 g $K_2HPO_4 \cdot 3H_2O$), 0.3 g $MgSO_4 \cdot 7H_2O$, and 15 g agar. This medium has a pH of 6.9 after autoclaving for 15 min at 20 lb./inch^2 at 120 C.

TABLE 1. Description of bacteria used on the D-series selective media

Species[a]	Strain no.	Source
Agrobacterium gypsophilae	ATCC 13329	M. P. Starr
A. pseudotsugae	ICPB TP3	M. P. Starr
A. radiobacter	22	A. Kerr
A. rhizogenes	Munnecke R136b	M. P. Starr
A. rubi	ATCC 13334	M. P. Starr
A. tumefaciens	6111 and 14 and 27	J. E. DeVay A. Kerr
Cornebacterium diphtheriae-gravis	ATCC 11049L2	E. L. Biberstein
C. equi	ATCC 1685	E. L. Biberstein
C. fascians		R. G. Grogan
C. michiganense	493	R. G. Grogan
C. pseudotuberculosis	B1689	E. L. Biberstein
C. pyogenes	B1724	E. L. Biberstein
C. renale	ATCC 1686	E. L. Biberstein
Erwinia amylovora	FB-10	M. N. Schroth
E. aroideae	EA-1	M. N. Schroth
E. carotovora	EC-1	M. N. Schroth
E. dieffenbachiae	ED-1	M. N. Schroth
E. nigrifluens	133	E. E. Wilson
E. quercina	Acorn-1	M. N. Schroth
E. uredovora	ICPB XU102	M. P. Starr
Escherichia coli	AB 295	A. J. Clark
Pseudomonas cichorii	NCPPB 668	R. A. Lelliot
P. glycinea	564	C. Leben
P. lachrymans		R. G. Grogan
P. marginalis	NCPPB 247	R. A. Lelliot
P. phaseolicola		R. G. Grogan
P. mors-prunorum	NCPPB 624	R. A. Lelliot
P. savastanoi	1006	E. E. Wilson
P. solanacearum	P28	I. Buddenhagen
P. syringae	B3	J. E. DeVay
P. tabaci		R. G. Grogan
P. viridiflava	NCPPB 1810	R. A. Lelliot
Rhizobium japonicum	RH 168	J. E. DeVay
R. leguminosarum	RH 34	J. E. DeVay
R. lupini	D 25	J. E. DeVay
R. meliloti	RH 6	J. E. DeVay
R. trifolii	RH 36	J. E. DeVay
Salmonella typhimurium	LT-2	J. Ingraham
Xanthomonas alfalfae	XA-129	W. C. Schnathorst
X. cyamopsidis		R. G. Orellana
X. malvacearum	Race 1	W. C. Schnathorst
X. pelargonii	XP-27	W. C. Schnathorst
X. phaseoli	XP-2	W. C. Schnathorst
X. pruni	XP-11	W. C. Schnathorst
X. translucens	XT-104	W. C. Schnathorst
X. vesicatoria		R. G. Grogan

[a] All other species not listed are those of our laboratory culture collection.

Medium D1 (for *Agrobacterium*) contains per liter: 15 g mannitol, 5 g $NaNO_3$, 6 g LiCl, 20 mg $Ca(NO_3)_2 \cdot 4H_2O$, 2 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g bromthymol blue, and 15 g agar. The medium has a pH of 7.2 after autoclaving and should appear dark blue.

Medium D2 (for *Corynebacterium*) contains, per liter: 10 g glucose, 4 g casein hydrolysate, 2 g yeast extract, 1 g NH_4Cl, 0.3 g $MgSO_4 \cdot 7H_2O$, 5 g LiCl, 1.2 g Tris, 40 mg polymyxin sulfate (300 units), 2 mg sodium azide, and 15 g agar. The medium is adjusted with HCl to pH 7.8 before autoclaving. Polymyxin sulfate and sodium azide are added after the medium has cooled to about 50 C after autoclaving. This medium has a pH of 6.9 after autoclaving. The medium should be freshly prepared, since azide and polymyxin break down with time.

Medium D3 (for *Erwinia*) contains, per liter: 10 g sucrose, 10 g arabinose, 5 g casein hydrolysate, 7 g LiCl, 3 g glycine, 5 g NaCl, 0.3 g $MgSO_4 \cdot 7H_2O$, 50 mg sodium dodecyl sulfate, 60 mg bromthymol blue, 100 mg acid fuchsin, and 15 g agar. The medium is adjusted to pH 8.2 with NaOH before autoclaving. The medium has a pH of 6.9-7.1 after autoclaving.

Medium D4 (for *Pseudomonas*) contains, per liter: 10 ml glycerol, 10 g sucrose, 1 g casein hydrolysate, 5 g NH_4Cl, 2.3 g Na_2HPO_4, 0.6 g sodium dodecyl sulfate, and 15 g agar. The medium has a pH of 6.8 after autoclaving.

Medium D5 (for *Xanthomonas*) contains, per liter: 10 g cellobiose, 3 g K_2HPO_4, 1 g NaH_2PO_4, 1 g NH_4Cl, 0.3 g $MgSO_4 \cdot 7H_2O$, and 15 g agar.

PM buffer, pH 7.25, contains, per liter: 7 g K_2HPO_4, 2 g KH_2PO_4, and 250 mg $MgSO_4 \cdot 7H_2O$. Saline contains 8.5 g NaCl/liter.

Medium B was prepared as outlined by King et al. (13). Yeast extract-glucose-calcium carbonate agar, known as YGC or YDC, was prepared by the method of Dowson (7), except that glucose was autoclaved separately and ultrafine precipitated calcium carbonate (Mallinkrodt Chemical Works) was used. This avoids the formation of the heavy white precipitate that usually accumulates at the bottom of culture dishes and tubes when other calcium carbonates are used. Nutrient agar was prepared according to the Difco Manual (6).

RESULTS.—*Replica plating*.—The D-series media were tested with representative species of various genera for the extent and range of selectivity. Colonies representing each of the species listed (Table 2) developed on medium 523 as expected for a general medium (Fig. 1-A), and selective growth on the D-series media (Fig. 1) was as follows:

1) On medium D1, *Agrobacterium* species grew but growth of other genera was inhibited (Fig. 1-B). Colonies of *Agrobacterium* are usually circular, convex, and glistening. Initially they appear light blue, then turn dark olive-green. *Agrobacterium tumefaciens* (No. 27) obtained from A. Kerr did not grow on this medium nor on a medium designed by Schroth et al.

TABLE 2. Species were placed in the following template positions on selective D-series media[a]

Agrobacterium, *Rhizobium* spp.	*Corynebacterium* spp.[c]	*Erwinia*, *Escherichia*, *Salmonella* spp.	*Pseudomonas* spp.	*Xanthomonas* spp.
Agrobacterium tumefaciens[b,g]	*Corynebacterium flaccumfaciens*	*Erwinia amylovora*[d]	*Pseudomonas savastanoi*[f]	*Xanthomonas malvacearum*[g]
A. gypsophilae[b,d]	*C. insidiosum*	*E. nigrifluens*[c]	*P. lachrymans*[f]	*X. campestris*[g]
A. rhizogenes[b,g]	*C. michiganense*	*E. rubrifaciens*[d]	*P. phaseolicola*[f]	*X. juglandis*[g]
A. pseudotsugae[b]	*C. poinsettiae*	*S. typhimurium*[d,f]	*P. syringae*[f]	*X. cyamopsidis*[d,g]
A. rubi[b,g]	*C. sepedonicum*	*Escherichia coli*[d,f]	*P. viridiflava*[b,f]	*X. fragariae*[g]
R. trifolii[g]	*C. fascians*	*E. aroideae*[d,f]	*P. marginalis*[d]	*X. vesicatoria*[g]
R. meliloti[g]	*C. equi*	*E. quercina*[d]	*P. cichorii*[f]	*X. pruni*[g]
R. leguminosarum[g]	*C. renale*	*E. cassavae*[c]	*P. solanacearum*[e]	*X. phaseoli*[g]
R. lupini[g]	*C. pseudotuberculosis*	*E. ananas*[c]	*P. glycinea*[f]	*X. transluscens*[g]
R. japonicum[g]	*C. pyogenes*	*E. uredovora*[d,g,f]	*P. mors-prunorum*[f]	*X. pelargonii*[g]
Agrobacterium radiobacter[b,g]	*C. diphtheriae*	*E. carotovora*[d]	*P. mori*[f]	*X. alfalfae*[g]
		E. dieffenbachiae[d]	*P. tabaci*[d,f]	

[a] All the species listed were replicated as shown in the above order on medium 523, D1, D2, D3, D4, and D5. List of bacteria corresponds to growth on these selective media as shown in Fig. 1. Medium 523 permits growth of all bacteria listed and is a general media for plant pathogenic bacteria. Medium D1 selects for *Agrobacterium* species; medium D2 for *Corynebacterium* species; medium D3 for *Erwinia* species; medium D4 for *Pseudomonas* species; and medium D5 for *Xanthomonas* species.
[b] Growth on medium D1. *P. viridiflava* grew very poorly.
[c] Growth on medium D2. Note the characteristic spreading growth of *Erwinia* spp. in Fig. 1-C.
[d] Growth on medium D3. The pseudomonads grew poorly and stopped growth after 3 days.
[e] No growth on medium D4.
[f] Growth on medium D4.
[g] Growth on medium D5. Note the growth of species in columns 1 and 5 in Fig. 1-F.

(28). This isolate and some of our strains of *A. tumefaciens* will not grow since they are unable to utilize nitrate. *Agrobacterium rhizogenes* grows very poorly on medium D1. Colonies of *A. radiobacter* are indistinguishable from *A. tumefaciens*. *Rhizobium* species grow very slowly, requiring more than 7 days on this medium. Most of the species of the other genera listed in Table 1 did not grow. Residual growth and secondary colonies of a few species listed in Table 2 may appear when streaked heavily. This occurs on most selective media (1, 14, 25, 31, 35). When soil extracts were plated on this medium, the growth of saprophytic bacteria (mainly *Pseudomonas* species) is usually limited, but such colonies can be distinguished by the blue-to-yellow color change of the bromthymol blue around such colonies owing to the production of acid. Also, *Pseudomonas* saprophytes fail to absorb the dye. Bromthymol blue prevents growth of gram-positive bacteria, and lithium ions retard the growth of most *Pseudomonas* species. When medium D1 is used as an enrichment broth, both saprophytic *Pseudomonas* species (e.g., *P. fluorescens* and *P. denitrificans*) and *Agrobacterium* species will be enriched.

2) Medium D2 is selective for *Corynebacterium* species and certain "*Erwinia*" species, e.g., *E. ananas*, *E. cassavae* (Fig. 1-C). The colonies characteristic of *Corynebacterium* can be distinguished easily from these *Erwinia* species (Fig. 2), which are dull white or mucoid, flat, sometimes convexed, and spreading; those of *Corynebacterium* are small, light-yellow, circular, convex, and glistening. When soil and extracts of diseased tissue were plated, gram-positive cocci also grew on this medium.

Medium D2 supported good growth of the following *Corynebacterium* species pathogenic for man and animals: *C. equi*, *C. pseudotuberculosis*, *C. pyogenes*, and *C. renale*. *C. diphtheriae* required 5 days for good growth. Colonies were ovoid, shiny yellow-white, or light yellow. Growth appeared in 72 hr at 30 C.

3) Medium D3 is selective for *Erwinia* species (Fig. 1-D), which characteristically produce a red coloration of the medium; the intensity of the color depends on the species. Most of the soft-rotting group (*E. aroideae*, *E. atroseptica*, *E. carotovora*, etc.) characteristically produce a more intense color reaction than the amylovora group (e.g., *E. amylovora*, *E. quercina*, *E. tracheiphila*, *E. rubrifaciens*, etc.). This last group requires at least 48 hr before any reaction is noticeable. The color of the plate will eventually turn completely red-orange on prolonged growth of *Erwinia* species. The growth of other genera is usually suppressed, although some saprophytes (*Escherichia* species) may occasionally appear when soil is plated. Trials with unsterilized diseased tissues from naturally infected pear (fire blight) and walnut trees (bark canker) showed that this medium is selective for the respective causal organisms, *E. amylovora* and *E. rubrifaciens*. Some *Xanthomonas* species produce colonies after several days of incubation, but are easily distinguished by the dark-blue reaction surrounding their colonies and lack of strong acid production.

4) Medium D4 is selective for *Pseudomonas* species (Fig. 1-E). Most pseudomonads will rarely produce fluorescent pigments on this medium. *Pseudomonas solanacearum* did not grow on this medium. Certain *Erwinia* species will grow slowly if incubated on the medium for prolonged periods, or if an impure grade of sodium dodecyl sulfate is used. The efficiency of recovery is lower for this medium than for the others, but pseudomonads can be readily isolated because of its relatively high degree of selectivity.

5) Medium D5 favors growth of *Xanthomonas* and

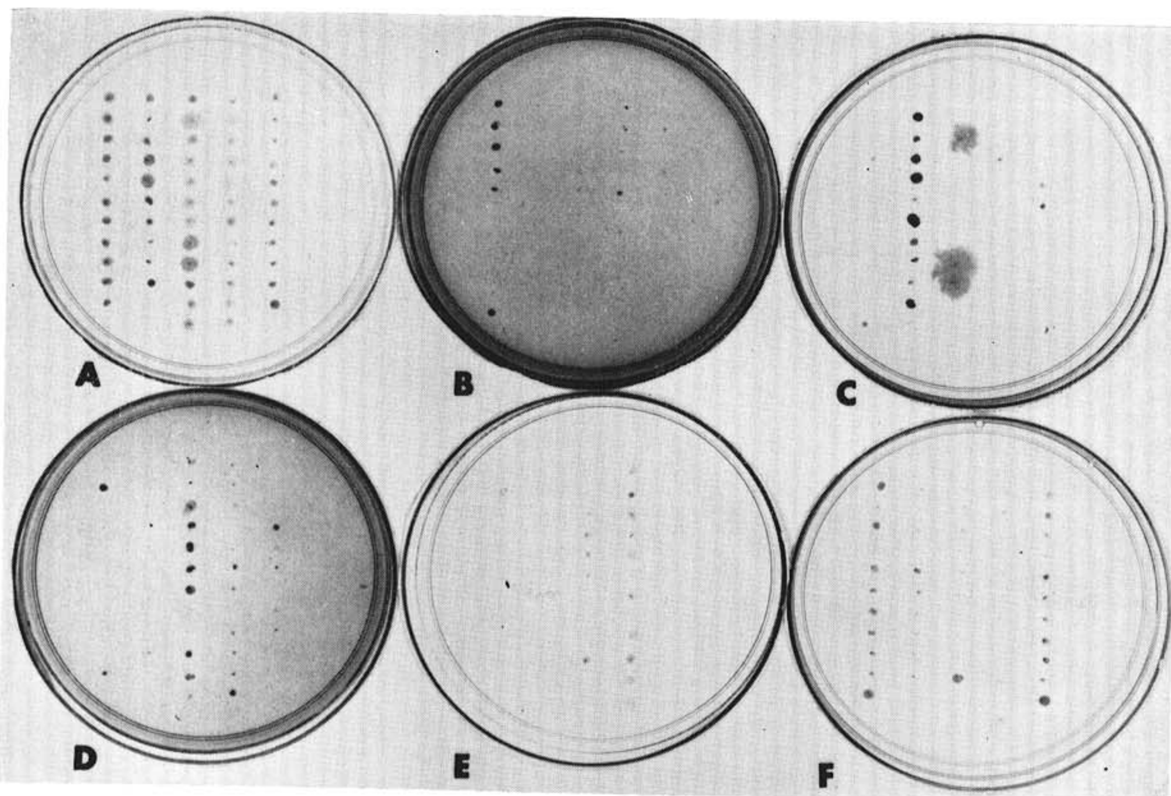

Fig. 1. Replicate plates of species listed according to Table 1. Plates show colony growth after incubating for 48 hr at 30 C. **A)** Medium 523 supports growth of all species. **B)** Medium D1 supports growth of six *Agrobacterium* species but no others. **C)** Medium D2 supports the growth of all *Corynebacterium* species, *Erwinia nigrifluens, E. cassavae,* and *E. ananas* (these *Erwinia* species were gram-variable and their pathogenicity unknown). **D)** Medium D3 supports the growth of all *Erwinia* species (except *E. nigrifluens, E. cassavae,* and *E. ananas*) and *Escherichia coli* B and *Salmonella typhimurium* LT-2. The *Erwinia* species are differentiated by the red-orange colored reactions on this medium. Growth of *A. gypsophilae* also occurs. **E)** Medium D4 supports the growth of all *Pseudomonas* species tested except *P. solanacearum,* and certain *Erwinia* species (*E. aroideae, E. ananas*) and *E. coli, S. typhimurium.* **F)** Medium D5 supports the growth of *Xanthomonas* species tested. With extended incubation, this medium will support the growth of *Agrobacterium* and *Rhizobium* and certain *Erwinia* species (*E. ananas*).

Agrobacterium species (Fig. 1-F), but the growth of *Pseudomonas* species is suppressed. Thus, *Xanthomonas* species can be readily isolated simply by plating crude extracts of the diseased tissue because saprophytic *Pseudomonas* species and some yellow-pigmented saprophytes (possibly *E. herbicola* types) are suppressed. *Agrobacterium* and *Xanthomonas* rarely occur together in nature; thus, the ability of both genera to grow on D5 presents no problem.

Growth on medium 523.—To estimate plating efficiencies, the average colony numbers of test organisms on nutrient agar (supplemented with 10 g/l glucose), King's medium B, and medium 523 were compared (Table 3). Few significant differences were found among the three media. Colonies of various species, however, were considerably larger on medium 523 than on nutrient agar and medium B, indicating that growth rates were greater on medium 523. Doubling times of *E. rubrifaciens* and *A. tumefaciens* were respectively 34 and 30 min in 523 broth at 30 C, and 96 and 60 min in nutrient broth.

Plating efficiencies and degree of selectivity.—The degree of selectivity of each medium was evaluated by colony counts derived from mixed populations. Log phase cultures of *A. tumefaciens, C. michiganense, E. carotovora, E. amylovora, P. syringae,* and *X. campestris* were harvested and washed twice with cold PM buffer. Each bacterium was suspended in cold saline and adjusted to a concentration of 10^6 cells/ml, except for *P. syringae* which was adjusted to 10^7 cells/ml; 1.0 ml of each suspension was pooled, mixed, and diluted in ice-cold saline to approximately 10^3 cells/ml, and 0.1 ml of the mixture was distributed on each medium. Colonies were counted after the plates were incubated for 48 hr at 30 C (Table 4, Fig. 2). All of the species recovered remained pathogenic. Medium D5, designed to select *Xanthomonas* species, also supports good growth of *A. tumefaciens.* Preliminary trials showed consistent recovery of *X. campestris* and *X. fragariae* from homogenized diseased leaf tissues; the latter pathogen is usually difficult to isolate on ordinary media.

Recovery efficiency from soil.—Unsterilized fresh Yolo sandy loam was mixed with bacteria as follows: to 1.0 g of soil was added 1.0 ml of a mixture containing 10^5 cells/ml of each of the bacteria listed in

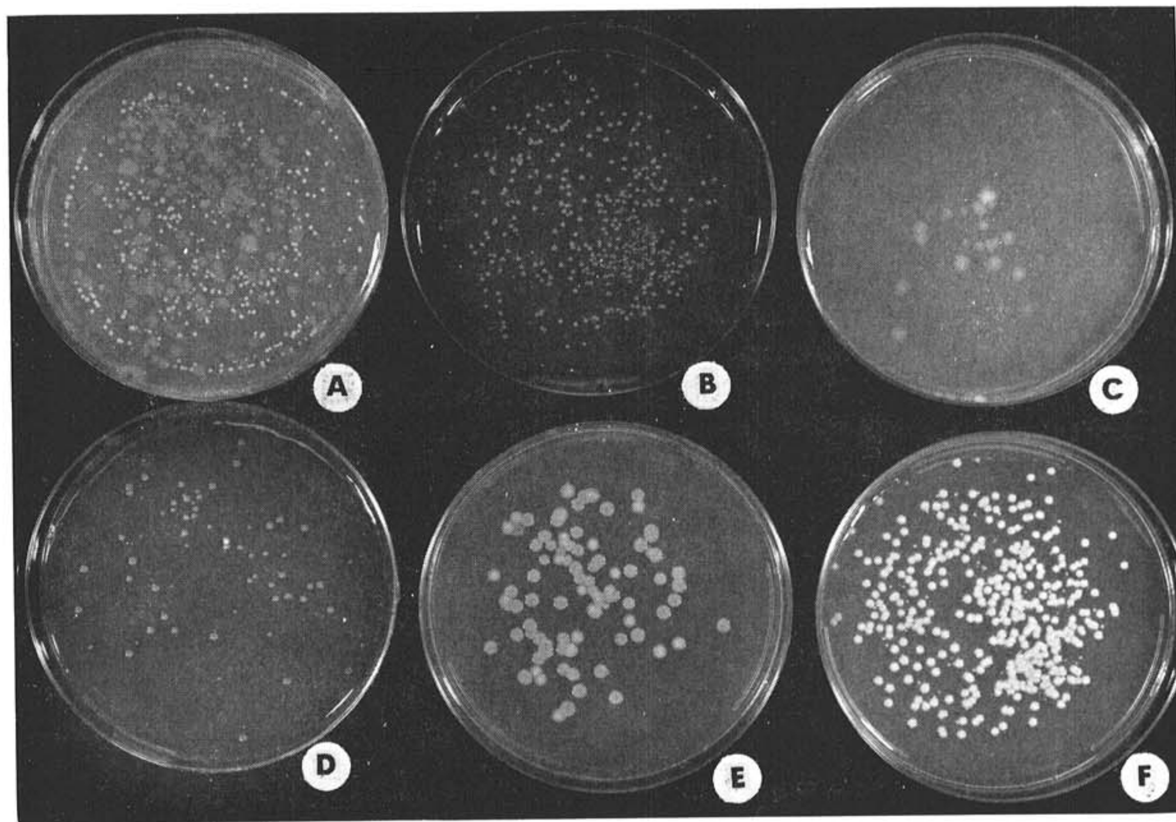

Fig. 2. Efficiency of recovery of various representative species from a mixed population of bacteria. Colony growth after 48 hr of incubation at 30 C. **A)** Mixture of colonies on medium 523. **B)** Growth of *Agrobacterium tumefaciens* 1D11 on medium D1. Colonies are usually light blue, clear, mucoid, circular, and convex. They later turn olive green. **C)** Growth of *Corynebacterium michiganense* 3D21 (small colonies) and *Erwinia carotovora* 3D3 (large colonies) on medium D2 (this *Erwinia* species was gram-variable and did not cause the usual soft rot symptoms on potato). *Corynebacterium michiganense* colonies are light yellow, circular, pulvinate, and usually small. *Erwinia carotovora* colonies are white, filamentous, flat, and spreading. **D)** *Erwinia amylovora* FB10 colonies on medium D3. Colonies are circular, convex, and bright red-orange. The color reaction around each colony is red-orange, with eventual spread of the color throughout the plate. **E)** *Pseudomonas syringae* B3 on medium D4. Colonies are raised slightly, circular, with erose margins. They are pale white and tinted slightly greenish. **F)** Growth of *Xanthomonas campestris* 2D5 and *Agrobacterium tumefaciens* 1D11 on medium D5. *Xanthomonas campestris* colonies are yellow, circular pulvinate, and mucoid. *Agrobacterium tumefaciens* are white, circular, convex, and mucoid.

Table 5. The soil slurry was diluted with 9.0 ml of saline, then mixed mechanically for 10 min. The suspension was diluted 1:10 in saline, and 0.1-ml portions were plated on D-series media. The efficiencies of recovering the plant-pathogenic bacteria from artificially infested soil is summarized in Table 5. Each medium permitted growth of only the plant-pathogenic bacterium for which it was designed. These recovered bacteria were pathogenic. Few, if any, soil bacteria grew on any of the D-series media, indicating that these media are highly selective and fungus colonies were not evident within the time normally required for growth of bacteria (2-4 days). These media have not all been tested on their effectiveness for selectively isolating plant-pathogenic bacteria from naturally infested soil. Only medium D1 and D3 have been employed extensively for isolating *A. tumefaciens* and *E. rubrifaciens* from field soils. A number of isolates of these species has been obtained from soil through the use of these media.

Recovery efficiency from diseased tissue.—The efficacy of the D-series media was tested on extracts made from naturally infected and experimentally infected plants. Samples were weighed and homogenized in saline in a mortar and diluted to 1% (w/v) with saline. Three 1-ml samples of the homogenized material were diluted in saline, and 0.1-ml samples were spread on the D-series plates. Colonies were counted after 48 hr at 30 C, and the counts and recovery efficiencies are summarized in Table 6. Each medium was selective for species in the genus for which the medium was designed, and permitted no growth of contaminating saprophytic bacteria.

Discussion.—From present studies with experimental and natural models, the D-series media appear sufficiently selective for isolating pathogens in the genera *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas,* and *Xanthomonas*. Fifty-seven species were tested (Table 2). Replication experiments indicate that separate media permitted growth of representative species

TABLE 3. Comparison of the growth of plant-pathogenic bacteria on various general-purpose media[a]

Bacteria	Colony no./plate		
	Nutrient agar + glucose	Medium B	Medium 523
Agrobacterium tumefaciens	292.3 ± 5.7[b]	299.0 ± 19.5	288.2 ± 18.1
Corynebacterium michiganense	169.7 ± 1.7	179.8 ± 2.1	156.4 ± 11.8
Erwinia amylovora	170.0 ± 5.6	196.0 ± 4.2	195.6 ± 5.9
Pseudomonas syringae	45.0 ± 2.0	50.0 ± 3.5	47.0 ± 2.2
Xanthomonas campestris	412.0 ± 24.8	376.0 ± 14.4	330.0 ± 17.4

[a] Cultures in log phase of each species were grown in 523 broth. They were washed twice in PM buffer and diluted with saline to approximately 10^3 cells/ml. Samples (0.1 ml) were plated in triplicate and the plates were then incubated for 48 hr at 30 C.

[b] Figures represent avg colony no. and standard error of the mean.

TABLE 4. Efficiency of recovery on selective D-series media of plant pathogens from a mixed population[a]

Bacteria	Selective medium	No. of colonies[b]	Efficiency[c] (%)
Agrobacterium tumefaciens	D1	267.4 ± 15.9	90.3
Corynebacterium michiganense	D2	63.8 ± 5.6	27.1
Erwinia amylovora	D3	138.1 ± 9.7	77.7
Pseudomonas syringae	D4	19.3 ± 3.9	6.5
Xanthomonas campestris	D5	106.9 ± 3.2	78.4

[a] D-series media represent a group of selective media of which a given medium is selective for species in one given genus, i.e., medium D1 permits growth of *Agrobacterium* species, medium D2 selects for *Corynebacterium* species, etc. A mixture of the listed bacteria was prepared as follows: Cells of each species in exponential growth were harvested, washed twice in saline, and adjusted to 10^6 cells/ml. *P. syringae* was adjusted to 10^7 cells/ml. One ml of each species was then mixed together and diluted to give approximately 150 colonies/plate.

[b] Figures are avg colony no./plate from nine plates and standard error of the mean.

[c] $$\% \text{ Efficiency} = \frac{\text{no. colonies recovered on selective medium}}{\text{no. colonies on medium 523}} \times 10^2.$$

TABLE 5. Efficiency of the D-series media of recovering plant-pathogenic bacteria from soil[a]

Bacteria	Selective medium	No. colonies[b]	Efficiency[c] (%)
Agrobacterium tumefaciens	D1	128.3 ± 5.5	48.1
Corynebacterium michiganense	D2	51.0 ± 4.5	79.8
Erwinia amylovora	D3	48.5 ± 8.6	57.0
Pseudomonas syringae	D4	52.8 ± 9.8	26.9
Xanthomonas campestris	D5	85.0 ± 12.4	79.9

[a] The D-series media are described in the footnote of Table 3. Soil: Yolo sandy loam containing 2.7% free water (as opposed to chemically bound water), 7.4 mg/g organic material (sucrose equivalent), and 5 mg/g N (NH_4^+ form). Unsterilized soil (1.0 g) was mixed with 1.0 ml of 10^5 cells/ml of each listed bacterium. The mixture was diluted 1:10, and 0.1-ml portions were plated on each medium listed. Similar dilutions were made of each species without soil and plated on the media listed that was designed for the species, e.g., medium D1 for *A. tumefaciens*, D2 for *C. michiganense*, etc. The plates were incubated for 48 hr at 30 C.

[b] Figures represent colony no./plate in nine plates and standard error of the mean.

[c] $$\% \text{ Recovery} = \frac{\text{no. colonies from soil}}{\text{no. colonies on the medium designated for the representative species assigned}} \times 10^2.$$

(after 2 to 4 days) of each genus (Fig. 1). As with various known differential media (e.g., EMB), pathogenic species might be differentiated by their distinctive colony characteristics on the D-series media. There are a number of apparent advantages in using these media. First, each medium is designed to select species of one particular genus of bacteria; thus labor, material, and time is minimized; second, each medium is useful for studying the ecology of a given bacterium, e.g., medium D3 for determining the survival and distribution of *E. amylovora*; third, each medium can be used for quantitating a particular bacterium in nature with appreciable accuracy, since the plating efficiency of each medium has been calculated; and finally, these media are defined and can be made easily.

The degree of selectivity of the D-series media may not apply to all strains of phytopathogenic bacteria. Also, colonies of mutants resistant to the inhibitory constituents in the media will invariably develop if the selective media are streaked heavily or if used in the liquid form for enrichment purposes. This was demonstrated consistently by seeding the liquid form of medium D1 with unsterilized soil and incubating for several days. Usually saprophytic pseudomonads were enriched. Plant-pathogenic pseudomonads were rarely enriched. Secondary colonies are known to arise on other selective media when streaked with heavy suspensions of bacteria (1, 14, 25, 31, 35), a condition which does apply when isolations are made of infested or infected material. This was noted by D. C. Hildebrand & M. N. Schroth (*personal communication*) from independent tests with our media.

The design of the D-series media (except for medium D5) was based on altering the membranes and cell wall or capsular components so that differential permeability is reduced or abolished. Permeability of the membrane is a function of carrier proteins specific for distinct molecules and ions in the medium (21). The cell wall is preferentially permeable by serving as a molecular sieve (23). The normal functions and synthesis of these structural components were therefore modified or inhibited by using sodium dodecyl sulfate (lipoproteins and polysaccharides) (8, 9), lithium ions (5) and polymyxin (membranes) (29), and glycine (cell wall synthesis) (5, 18). Lithium chloride may also affect protein synthesis, since it inhibits

TABLE 6. The efficacy of media in recovery of plant-pathogenic bacteria from diseased tissue

Bacterium and source	Selective medium	Tissue source[a]	Recovery[b] (colonies/g)
Agrobacterium tumefaciens no. 14 (Kerr)	D1	Sunflower (gall)	$50.3 \pm 2.8 \times 10^4$
A. tumefaciens 1D11 (Kado)	D1	Sunflower (gall)	$20.0 \pm 1.0 \times 10^8$
Corynebacterium michiganense 493 (Grogan)	D2	Tomato (stem)	$30.6 \pm 3.5 \times 10^7$
C. michiganense 3D2 (Kado)	D2	Tomato (stem)	$41.6 \pm 4.8 \times 10^5$
Erwinia carotovora EC-1 (Schroth)	D3	Potato (tuber)	$26.7 \pm 1.8 \times 10^7$
E. carotovora (Segall)	D3	Potato (tuber)	$18.0 \pm 0.4 \times 10^5$
E. amylovora 1D3 (Kado)	D3	Apple (twig)	$13.4 \pm 0.5 \times 10^7$
E. amylovora 1D3 (Kado)	D3	Apple (twig)	$46.0 \pm 5.3 \times 10^6$
Pseudomonas syringae B3 (DeVay)	D4	Peach (stem)	$9.0 \pm 2.6 \times 10^3$
Xanthomonas campestris 2D5 (Kado)	D5	Chinese cabbage (leaf)	$33.7 \pm 2.9 \times 10^5$
X. campestris 2D5 (Kado)	D5	Chinese cabbage (leaf)	$15.0 \pm 1.0 \times 10^7$

[a] From experimentally inoculated material.
[b] Figures represent avg no. colonies/g of diseased tissue (fresh wt) and standard error of the mean. Recovered bacteria were pathogenic.

the binding of aminoacyl transfer RNA to ribosomes (33). These and other compounds that principally affect the cell's outer components, combined with bactericidal agents, may be useful in controlling plant diseases because the sensitized pathogen is affected by lower-than-usual concentrations of bactericidal agents. An example is medium D2, which contains polymyxin and azide; neither bacteriocide is effective when used alone; together, they eliminate growth of most gram-negative bacteria. Also, plant-pathogenic bacteria such as *E. amylovora* were made extremely sensitive to streptomycin at 1:1,000, the lethal antibiotic concentration, simply by the addition of sodium dodecyl sulfate (Kado, *unpublished data*).

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