# A Semiselective Agar Medium for Isolation of *Clavibacter michiganensis* subsp. *sepedonicus* from Potato Tissues

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**ABSTRACT**

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A new semiselective agar medium, NCP-88, was developed for isolating *Clavibacter michiganensis* subsp. *sepedonicus*, the cause of bacterial ring rot, from infected potato plant parts. Important selective components of this nutrient agar, yeast extract, and salts medium are mannitol, polymyxin B-sulfate, nalidixic acid, and cycloheximide. NCP-88 permitted a better balance between plating efficiency and selectivity than did other reported semiselective media. In tests of 21 strains of *C. m. sepedonicus* from pure culture, NCP-88 supported 75–133% of the colony numbers that developed on nutrient broth yeast extract agar (NBY). Overall recovery of the pathogen from stem and tuber tissues of symptomatic or asymptomatic potato plants from Idaho and Colorado was significantly greater with NCP-88 than with NBY. Approximately 79% of the total nontarget bacterial population recovered from potato tissues with NBY did not grow on NCP-88 during the 7-day incubation period. Gram-positive nontarget coryneforms on NCP-88 could be distinguished from *C. m. sepedonicus* by colony morphology and pigmentation. Antagonistic gram-negative bacteria grew on NBY and often prevented the growth of *C. m. sepedonicus*. These antagonists were sufficiently suppressed on NCP-88 to allow isolation of *C. m. sepedonicus*. No fungal growth was observed on NCP-88 throughout the 7-day incubation period at 23 C. The new semiselective medium shows promise as an improved tool for detection of viable cells of *C. m. sepedonicus* in symptomatic and asymptomatic potato plants.

*Clavibacter michiganensis* subsp. *sepedonicus* (Spieck. and Kott.) Davis et al (syn. *Corynebacterium sepedonicum* Skap. and Burkh.) causes bacterial ring rot of potato. The disease is important especially in seed potato production because a zero tolerance has been imposed by regulatory agencies in the United States, Canada, and Europe (6). In North America, ring rot is responsible for about 60% of all seed potato acreage rejected annually for certification (29). Detection of ring rot is based primarily on symptomatology and one or more confirmatory tests including Gram's stain (15), serology (2–5,7,8,23,29,30), and pathogenicity on eggplant (1,18). Each of these tests has limitations such as low specificity, unavailability of materials, and/or excessive time requirements (28). Direct isolation of *C. m. sepedonicus* from potato tissues would be useful for ring rot diagnosis, but this procedure is not normally done because of the lack of a reliable semiselective medium (28,35).

Development of semiselective media for coryneforms such as *C. m. sepedonicus* often is difficult because of their fastidious nature and inherent susceptibility to antibiotics and inhibitors. Although the nutritional requirements of *C. m. sepedonicus* have been studied (13,16, 17,19,26,33), results were inconclusive and sometimes conflicting. This may be attributable to differences in nutritional requirements among strains. Starr (33) showed that *C. m. sepedonicus* cannot grow well on basic synthetic medium alone and that additional undefined components such as casein hydrolysate or yeast extract are necessary for growth. Furthermore, the pathogen seemed to be limited in its ability to utilize carbon sources (20). Although arabinose, xylose, galactose, and levulose are useful carbon sources, glucose is used most often to culture *C. m. sepedonicus* (14,20,22,32). Mannitol apparently is the only sugar alcohol utilized by the bacterium (32).

Inhibitors such as lithium chloride and polymyxins that may alter surface components and cell membranes are usually tolerated by coryneforms and are useful in suppressing the growth of gram-negative microbes (14). These inhibitors and sodium azide are the main components of the D-2 medium (14), which is selective for several coryneforms and some *Erwinia* spp. but permits the growth of gram-positive cocci. In contrast, M-80, which is semiselective for *C. m. sepedonicus* (20), contains LiCl, cycloheximide, sodium dichromate, and nalidixic acid. These chemicals apparently inhibit certain strains of gram-positive cocci, *Erwinia carotovora* subsp. *atroseptica* (van Hall) Dye, and *Pseudomonas fluorescens* (Trevisan) Migula. Many inhibitors were not included in M-80 because they either inhibited *C. m. sepedonicus* or were ineffective against nontarget organisms frequently associated with potato tissues such as *E. c. atroseptica* and *P. fluorescens* (20). In other studies, Sniezko and Bonde (32) recommended the addition of sodium dichromate to 4-d medium to prevent the "toxic effect of secondary invaders" against *C. m. sepedonicus*, whereas Marten et al (22) added potassium dichromate to inhibit *E. c. carotovora*. Potassium tellurite (10,24) and nalidixic acid (10,12) also are useful selective components of semiselective media for other coryneforms. However, most cationic and anionic agents, as well as various dyes and salts, offer little or no selective advantage for the growth of gram-positive bacteria (12).

The purpose of this study was to develop a sensitive and useful semiselective agar medium for isolating the ring rot bacterium from symptomatic and asymptomatic potato plants.

## MATERIALS AND METHODS

**Bacterial strains.** The origins, donors, and hosts of *C. m. sepedonicus* strains used in this study are shown in Table 1. Working cultures were stored in nutrient broth yeast extract (NBY) (34) agar slants at 4 C while stock cultures were maintained in NBY with 14% (v/v) glycerol at −80 C.

**Estimation of plating efficiency.** NBY was used as a standard for comparison in all tests because of its suitability as a cultivation medium for *C. m. sepedonicus* and other plant pathogenic coryneforms (35). In tests of plating efficiency from pure cultures, *C. m. sepedonicus* strains were grown on NBY slants for 4–5 days at 23 C, suspended in sterile saline solution (0.85% NaCl), and adjusted to $10^8$–$10^9$ cfu/ml based on spectrophotometric measurement of suspension turbidity. Serial dilutions were surface-plated in triplicate on test media. Colonies were counted after 7 days of incubation at 23 C and percent plating efficiency was calculated as (*C. m. sepedonicus* population on test medium/ *C. m. sepedonicus* population on NBY) × 100.

**Estimation of selectivity.** Unless otherwise specified, 1–5 g of either potato stems with roots or tuber tissues was macerated in a sterile mortar and 10 ml

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of 0.85% saline solution was added to the macerate. Serial dilutions were prepared, surface-plated on test media, and incubated as described above. Percent selectivity was computed as [(population of nontarget microbes on NBY − population of nontarget microbes on test medium)/population of nontarget microbes on NBY] × 100. Averages were derived from triplicate plates per dilution and three dilutions per sample.

**Development of semiselective agar medium.** NBY was modified by incorporating selected inhibitors and a carbon source. In a preliminary survey of 21 carbon sources, the addition of mannitol to NBY yielded the largest and most uniform *C. m. sepedonicus* colonies and the highest mean plating efficiency (percent recovery) of representative strains (9). The sensitivity of *C. m. sepedonicus* strains to 32 antibiotics and inhibitors applied to paper disks (27) also was tested. Polymyxin B-sulfate, nalidixic acid, Mueller's tellurite serum (24), and cycloheximide were selected because they were least inhibitory to representative strains. Optimal concentrations of these inhibitors were determined from plots of mean percent plating efficiency or colony diameter of *C. m. sepedonicus* against inhibitor concentration. The highest concentration that permitted at least 80% plating efficiency or 1.0-mm colony diameter (when inhibitors affected only colony size) was selected (9).

The final semiselective medium designated NCP-88 contained (g/L) Difco nutrient agar (23.0), yeast extract (2.0), $K_2HPO_4$ (2.0), $KH_2PO_4$ (0.5), $MgSO_4 \cdot 7H_2O$ (0.25), and D-mannitol (5.0). After sterilization, the medium was cooled to 50 C and filter-sterilized solutions of the following inhibitors were added per liter: 300 μl of polymyxin B-sulfate stock (7,900 units per milligram, 10 mg/ml stock), 800 μl of nalidixic acid stock (Na-salt, freshly dissolved in 10 mM NaOH, 10 mg/ml stock), and 2.0 ml of cycloheximide stock (dissolved in 47.5% ethanol, 100 mg/ml stock). Mueller's tellurite serum was initially incorporated at 1.0 ml/L but was later deleted.

**Comparison with other semiselective media.** NCP-88 plus tellurite (NMCP-88) was compared to four other media previously reported to be selective for *C. m. sepedonicus*—M-80 (20), D-2 (14), 4-d medium with sodium dichromate at 0.05 g/L (32), and the medium of Marten et al (22). Plating efficiency of 20 *C. m. sepedonicus* strains on these test media was determined using the procedures described above. Percent selectivity was determined from three samples of potato stem and root macerates. Means were compared using Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$.

**Isolation of *C. m. sepedonicus* from potato tissues.** Stems were collected from potato plants grown from seed tubers that had been artificially inoculated and planted in San Luis Valley, CO. About 60–70% of these stem samples came from plants that had typical ring rot symptoms. Tuber samples were also collected from Colorado but only from symptomless plants. Additional stem samples were obtained from Idaho from naturally infected field grown potato plants showing apparent ring rot symptoms. Idaho samples were previously tested serologically for ring rot by the Idaho Crop Improvement Association using a double diffusion test and polyclonal antibodies.

Samples of aboveground and underground stems with roots (5- to 10-cm segments) were washed in running tap water, blotted on paper towels, and immersed in 95% ethanol for 1–2 min. Excess ethanol was blotted and then flamed. With tuber samples, the area surrounding the stolon attachment scar was wiped with a cotton swab soaked in 95% ethanol. Excess ethanol was flamed and tissues from this area were aseptically removed using a sterile scalpel or cork borer.

The sample tissues were macerated in a sterile mortar and mixed with 0.85% saline solution at a ratio of 10 ml of saline per 1–5 g of tissue. Serial dilutions of the macerate were plated on NCP-88 and on comparison media and incubated at 23 C for 7 days. Plating efficiency and

**Table 1.** Source of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) strains and plating efficiency on NCP-88 semiselective agar medium

| Strain | Origin | Donor | Host | Plating efficiency[y] (%) |
|---|---|---|---|---|
| *Cms*-Al | Alaska | A. de la Cruz | Potato | 75 |
| CS-20 | Canada | R. J. Copeman | Potato | 99 |
| R-1 | Canada | S. H. De Boer | Potato | 123 |
| R3 | Canada | S. H. De Boer | Potato | 100 |
| R-5 | Canada | S. H. De Boer | Potato | 86 |
| R-8 | Canada | S. H. De Boer | Potato | 100 |
| CO-19 | Colorado | A. de la Cruz | Potato | 95 |
| #7 | Idaho | B. Rogers | Potato | 91 |
| CSEP-3 | Idaho | B. Rogers | Potato | 86 |
| 3NR[z] | Idaho | C. Orser | Potato | 84 |
| CSEP-6 | Idaho | B. Rogers | Potato | 118 |
| CSEP-7 | Idaho | B. Rogers | Potato | 96 |
| CSEP-8 | Idaho | B. Rogers | Potato | 133 |
| CSEP-9 | Idaho | B. Rogers | Potato | 94 |
| CSK-1 | Maine | Unknown | Potato | 105 |
| MEK-1 | Maine | Unknown | Potato | 112 |
| NDCs-AS-1 | North Dakota | N. Gudmestad | Potato | 122 |
| NDCs-Off | North Dakota | N. Gudmestad | Potato | 79 |
| NDCsSB-65 | North Dakota | N. Gudmestad | Sugar beet | 112 |
| NDCsSB-Mono | North Dakota | N. Gudmestad | Sugar beet | 88 |
| CS-12 | New Jersey | R. J. Copeman | Potato | 94 |

[y] Average of three replicates per strain. Plating efficiency (%) = (*Cms* population on NCP-88/*Cms* population on nutrient broth yeast extract agar) × 100.
[z] Mutant strain of CSEP-3 resistant to rifampicin and nalidixic acid.

**Table 2.** Comparison of different semiselective media for plating efficiency of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) strains and inhibition of nontarget microbes (selectivity) associated with potato tissues

| Medium | Reference no. | Carbon source | Inhibitor(s) | Plating efficiency[x] (%) | Selectivity[y] (%) |
|---|---|---|---|---|---|
| NMCP-88 | This article | Mannitol | Nalidixic acid, Mueller's tellurite serum, cycloheximide, polymyxin B-sulfate | 88 b[z] | 72 b |
| D-2 | 14 | Glucose | LiCl, polymyxin sulfate, sodium azide | 0 c | 83 a |
| M-80 | 20 | Glucose | LiCl, cycloheximide, sodium dichromate, nalidixic acid | 114 a | 32 d |
| MLL | 22 | Glucose | Potassium dichromate | 84 b | 45 c |
| 4-d | 32 | Glucose | Sodium dichromate | 120 a | 13 e |

[x] Mean of 20 *Cms* strains with three replicates per strain. Plating efficiency (%) = (*Cms* population on test medium/*Cms* population on nutrient both yeast extract agar [NBY]) × 100.
[y] Mean of three replicates from a pooled sample of underground potato stems with roots. Selectivity (%) = [(population of nontarget microbes on NBY − population of nontarget microbes on test medium)/population of nontarget microbes on NBY] × 100.
[z] Means followed by different letter are significantly different using Fisher's protected LSD ($P < 0.05$).

selectivity were computed as described previously. The Wilcoxon's signed-rank procedure (11) was used to compare *C. m. sepedonicus* populations recovered on NCP-88 and on NBY.

**Pathogenicity test on eggplants.** Presumptive colonies of *C. m. sepedonicus* growing on NCP-88 were purified. Two to three representative colonies from each sample were tested for pathogenicity on eggplants (*Solanum melongena* L.). One-week-old eggplant seedlings (cv. Black Beauty) grown in Sunshine Mix No. 1 (Cascade Seed Co., Spokane, WA) in 10-cm plastic pots were puncture-inoculated with a cell suspension of $10^8$ cfu/ml according to the procedures of Bishop and Slack (1).

## RESULTS

**Comparison with other semiselective media.** Although D-2 medium (14) markedly inhibited the growth of nontarget microbes, none of the *C. m. sepedonicus* strains (Table 1) grew on the medium (Table 2). In contrast, the plating efficiency of all strains was excellent on 4-d medium with sodium dichromate (32) and on M-80 medium (20), however, both media exhibited poor selectivity. NMCP-88, on the other hand, provided a favorable balance between plating efficiency and selectivity. However, when NMCP-88 was tested for recovery of *C. m. sepedonicus* directly from infected potato tissues, Mueller's tellurite serum proved to be excessively toxic. Therefore, this inhibitor was eliminated from the recipe in an attempt to better recover in vivo strains. This final medium was called NCP-88, referring to nalidixic acid, cycloheximide, polymyxin B-sulfate, and the year of development. The elimination of tellurite reduced the inhibition of nontarget microbes by about 15% but improved the plating efficiency of *C. m. sepedonicus* from 88% (Table 2) to nearly 100% (Table 1). Colonies of the pathogen were visible on NCP-88 within 5 days at 25 C and measured 0.5–1.5 mm in diameter after 7–10 days. The colonies were round to irregular with entire margins, white to cream in color, raised, and usually mucoid and glistening. After 10–12 days of incubation, colonies changed in color to pale yellow.

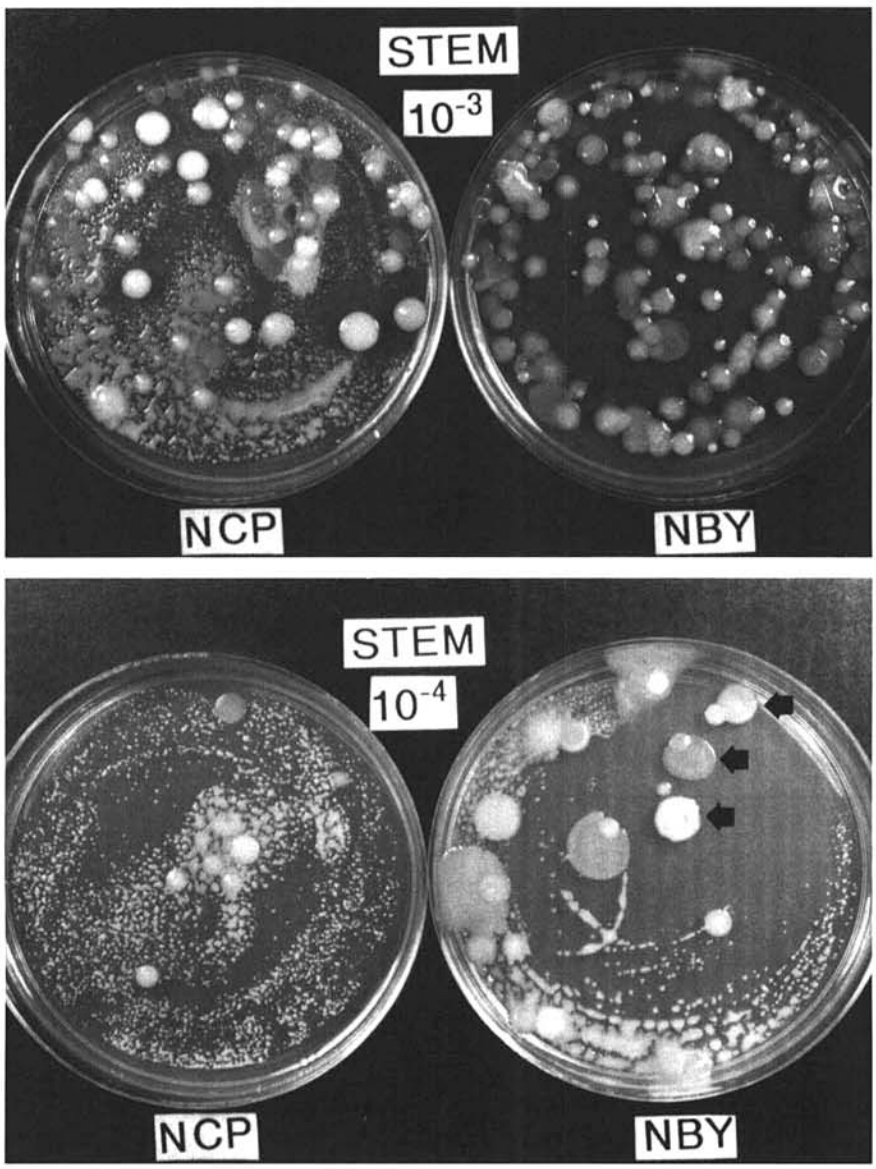


**Fig. 1.** *Clavibacter michiganensis* subsp. *sepedonicus* isolated from potato stems on NCP-88 (NCP) and nutrient broth yeast extract agar (NBY). Colonies of the pathogen are small, white, round to irregular, and slimy. Growth was from tissue macerate diluted to $10^{-3}$ (top) and $10^{-4}$ (bottom). Note the absence of *C. m. sepedonicus* colonies on NBY at $10^{-3}$ and the presence of large antagonistic colonies inhibiting *C. m. sepedonicus* growth at $10^{-4}$ dilution (arrows). Cultures were grown at 23 C for 7 days.

**Isolation of *C. m. sepedonicus* from potato tissues.** Eight of 27 underground stem samples from Idaho plants with apparent ring rot symptoms yielded *C. m. sepedonicus* colonies on NCP-88. Seven of these eight samples yielded *C. m. sepedonicus* colonies on NBY. All eight samples were positive in serological tests, as were three additional samples from which *C. m. sepedonicus* was not recovered on either medium. The pathogen population (cfu per gram of tissue) recovered from Idaho samples ranged from not detected to $7.6 \times 10^9$ on NBY and from $1.1 \times 10^9$ to $9.3 \times 10^9$ cfu/g of tissue on NCP-88. The average plating efficiency on NCP-88 relative to NBY was 123%.

Among 17 Colorado stem samples from plants with ring rot symptoms, 16 were positive on NCP-88, and 15 were positive on NBY. One of the 17 samples was negative on both NBY and NCP-88. *C. m. sepedonicus* populations (cfu per gram of tissue) recovered from 16 positive samples ranged from not detected to $1.4 \times 10^9$ on NBY and from $1.6 \times 10^6$ to $2.1 \times 10^9$ on NCP-88. Mean plating efficiency on NCP-88 relative to NBY was 333%. Six of 16 tuber samples from symptomless plants yielded *C. m. sepedonicus* on NCP-88, whereas five yielded *C. m. sepedonicus* on NBY. *C. m. sepedonicus* was not recovered with either NBY or NCP-88 from 10 of the 16 samples. The pathogen population (cfu per gram of tissue) recovered from tuber samples ranged from not detected to $1.0 \times 10^9$ on NBY and from $3.0 \times 10^5$ to $1.2 \times 10^9$ on NCP-88. The mean plating efficiency of NCP-88 relative to NBY was 195%. Overall recovery of the pathogen from stem and tuber samples from Idaho and Colorado was significantly greater on NCP-88 than on NBY according to the Wilcoxon's signed-rank procedure ($P < 0.05$).

Nontarget bacterial colonies that developed from the Idaho and Colorado samples grew in significantly higher numbers on NBY ($6.5 \times 10^4$ to $9.5 \times 10^8$ cfu/g of tissue) than on NCP-88 (undetected to $7.1 \times 10^8$ cfu/g of tissue). In some samples, no nontarget microbes grew on NCP-88, whereas high populations ($10^4$–$10^6$ cfu/g of tissue) were recovered on NBY. Overall selectivity of NCP-88 relative to NBY ranged from 25% to more than 99% with a mean at 79%.

Most of the nontarget bacteria that grew on NCP-88 were gram-positive cocci, short rods, or pleomorphic rods. Sixty percent of these were pigmented (shades of pink, orange, and yellow) and could be easily distinguished from the typical white to cream color of *C. m. sepedonicus* colonies. The cell size and morphology and the colony pigmentation and morphology of these nontarget bacteria closely resembled *Micrococcus, Arthrobacter*, and *Rhodococcus* spp. Only 20% of the nontarget bacteria that grew on NCP-88 were gram-negative. No fungal contaminants grew on NCP-88 throughout the 7-day incubation period.

Some nontarget bacteria that grew on NBY formed large inhibition zones against *C. m. sepedonicus* and negatively affected the recovery of the pathogen on this medium (Fig. 1). In some samples, these antagonists were numerous enough to prevent *C. m. sepedonicus* from growing on NBY regardless of the dilution that was plated. When these antagonists were few, *C. m. sepedonicus* could be recovered on NBY but only at higher dilutions such as $10^{-4}$–$10^{-6}$. In contrast, the suppression of these antagonists on NCP-88 permitted the recovery of *C. m. sepedonicus* even at lower dilutions. Other nontarget microbes extracted from potato tissues were not directly antagonistic to *C. m. sepedonicus* but tended to overgrow the pathogen on NBY plates (Fig. 2).

The bacteria that were antagonistic to *C. m. sepedonicus* on NBY were species of fluorescent pseudomonads. The most antagonistic strain, IS-1, was identified as *Pseudomonas aureofaciens* Kluyver based on morphological and biochemical characteristics and fatty acid analysis (9). IS-1 consistently produced inhibition zones larger than 50 mm in diameter on NBY plates. Two other prominent antagonists that were less inhibitory than IS-1 were identified as *P. aureofaciens* (IS-2) and *P. fluorescens* (IS-3). When pure cell suspensions ($10^{9}$–$10^{10}$ cfu/ml) of IS-1, IS-2, and IS-3 were separately plated on NCP-88 and on NBY, no IS-1 colonies grew on NCP-88 even after 7 days at 23 C. Populations of IS-2 and IS-3 were inhibited by more than 99% on NCP-88 relative to NBY. Furthermore, colonies of IS-2 and IS-3 were visible after 1 day on NBY but required 3–4 days to be visible on NCP-88.

In comparison with M-80 medium, NCP-88 clearly exhibited greater plating efficiency and selectivity (Table 3). All pathogenicity tests on eggplant with presumptive *C. m. sepedonicus* colonies recovered on NCP-88 were positive. Within 6–14 days from inoculation, eggplants developed typical symptoms characterized by marginal wilting of the first and second true leaves followed by chlorosis or necrosis.

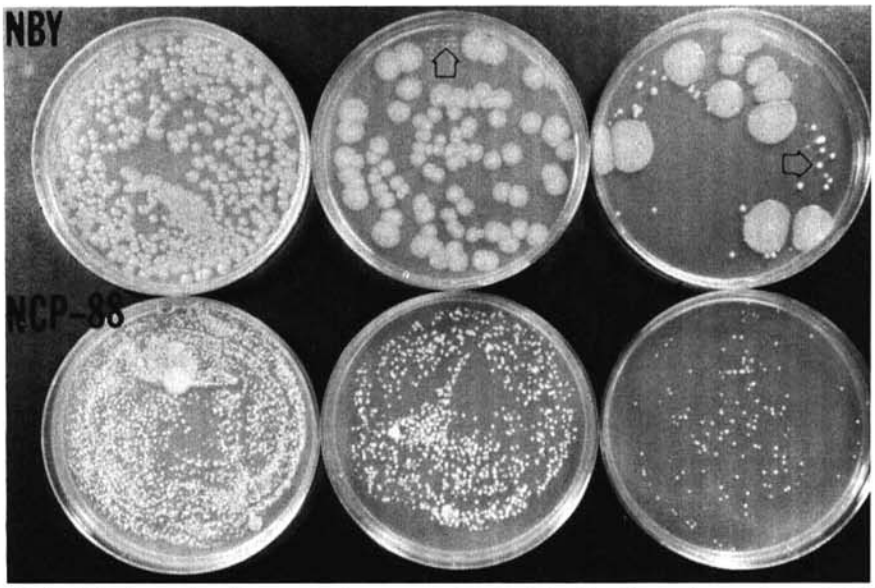


**Fig. 2.** Dilution series of a potato stem macerate on nutrient broth yeast extract agar (NBY) (top) and on NCP-88 (bottom) starting from $10^{-3}$ (left) to $10^{-5}$ (right). Note the abundant growth of *Clavibacter michiganensis* subsp. *sepedonicus* and the presence of few nontarget microbial colonies on NCP-88 compared to NBY. On NBY, *C. m. sepedonicus* colonies grew only at higher dilutions (arrows), whereas large colonies of nontarget microbes predominate. Cultures were grown at 23 C for 7 days.

**Table 3.** Comparison of plating efficiency and selectivity of NCP-88 and M-80

| Sample no.[x] | Plating efficiency[y] (%) | | Selectivity[z] (%) | |
|---|---|---|---|---|
| | NCP-88 | M-80 | NCP-88 | M-80 |
| 1 | 72 | 35 | 97 | 95 |
| 2 | 55 | 26 | 73 | 28 |
| 3 | 329 | 0.4 | 62 | 33 |
| 4 | ND | ND | 94 | 28 |
| 5 | ND | ND | 99 | 92 |

[x] Underground stem samples from Idaho.

[y] Mean of three plates per sample based on one out of three dilutions giving countable colonies. Plating efficiency (%) = (*Cms* population on test medium/*Cms* population on nutrient broth yeast extract agar [NBY]) × 100.

[z] Mean of three plates per sample based on one out of three dilutions giving countable colonies. Selectivity (%) = [(population of nontarget microbes on NBY − population of nontarget microbes on test medium)/population of nontarget microbes on NBY] × 100.

## DISCUSSION

Developing an improved semiselective agar medium for *C. m. sepedonicus* was difficult because of the fastidious and slow-growing nature of this pathogen. Initial efforts to develop a synthetic medium failed because of the complex nutritional requirements of *C. m. sepedonicus* and the variability of such requirements among strains (9). Only a few microbial inhibitors were useful because of the general sensitivity of *C. m. sepedonicus* to these compounds. Initially, Mueller's tellurite serum appeared to be useful as selective component but was later eliminated from the final semiselective medium because of its toxicity to wild (freshly isolated) strains. Apparently, *C. m. sepedonicus* strains that have been maintained in laboratory media have altered physiological characteristics. For example, the increased production of polysaccharide slime in vitro could be responsible for increased tolerance to inhibitors. In similar studies, Smidt and Vidaver (31) observed that LiCl was toxic to freshly isolated *C. m. nebraskense* (Vidaver and Mandel) Davis et al, thus, elimination of this selective component from the semiselective medium was suggested.

Unlike previous semiselective media (14,20,22,32), NCP-88 uses mannitol rather than glucose as a carbon source because of its more selective support of *C. m. sepedonicus* growth (9). In related studies, Magnuson (20) also observed the rapid growth of *C. m. sepedonicus* on mannitol but rejected it because of its incompatibility with certain other selective components in M-80.

Among the selective components of NCP-88, polymyxin B-sulfate appeared to be most useful in inhibiting nontarget microbes because elimination of this antibiotic from the medium resulted in a considerable decrease in selectivity (9). In particular, polymyxin may be principally responsible for suppressing the growth of the pseudomonads that suppress the growth of *C. m. sepedonicus* on NBY. However, the concerted action of all selective components in NCP-88

appears to be responsible for the overall inhibition of nontarget microbes. Other scientists noted the effectiveness of polymyxin against pseudomonads (25), but this antibiotic must be combined with sodium azide to eliminate most gram-negative bacteria (14). Neither polymyxin nor sodium azide was effective individually against these nontarget microbes (14).

Because antagonistic bacteria grow rapidly on NBY, this and other nonselective media should not be used for ring rot diagnosis because of the high risk of obtaining false negative results. NCP-88 is better suited for this purpose because antagonistic bacteria and other nontarget microbes are suppressed. In any case, surface-sterilization of host tissues is advisable if not necessary to eliminate most resident contaminant microorganisms before *C. m. sepedonicus* isolation on NCP-88. Also, the semiselective medium should be freshly prepared to avoid any possible breakdown of selective components, especially polymyxin (14). Furthermore, NCP-88 may not be very useful for isolating the pathogen from complex microbial environments such as in soil or in decaying plant tissues.

Tissue macerate dilutions of $10^{-2}$ or greater should be plated on NCP-88 since it may be difficult to detect *C. m. sepedonicus* in lower dilutions where nontarget microbes are abundant. Thus, the estimated minimum detection level of *C. m. sepedonicus* with NCP-88 is between $10^4$ and $10^5$ cfu/g of plant tissue (fresh weight). Magnuson (20) reported that dilutions of $10^{-5}$ or higher from a suspension of 0.1 o.d. of tuber exudate must be plated on M-80 to successfully isolate the pathogen. No minimum detection levels are available for D-2, 4-d, or cited by Marten et al (22). However, Magnuson (20) was not able to recover *C. m. sepedonicus* from infected tubers on D-2. For serological double diffusion and latex agglutination tests, about $2.0 \times 10^7$ cells per milliliter are required for visible agglutination to occur (29). Latex agglutination requires a minimum of $10^6$ cells per milliliter (30), whereas indirect fluorescent antibody stain can detect $10^1$–$10^2$ cells per milliliter (29). On the other hand, the Gram's stain procedure can detect a minimum of $10^2$ cells per milliliter (29). Besides sensitivity, NCP-88 offers the reliability and efficiency to make it a useful diagnostic tool.

In various tests with NCP-88, we found that *C. m. sepedonicus* is not always present in plants with apparent ring rot symptoms and not all symptomless plants are free of *C. m. sepedonicus*. In three of 27 cases, positive agglutination resulted in the double diffusion test, whereas no *C. m. sepedonicus* colonies were detected on NCP-88. The presence of *C. m. sepedonicus* in these three samples is debatable since cross reactivity is a problem in ring rot serology (3,4). Also, nonviable bacterial cells may yield positive serological tests.

These observations and that of others (21) suggest that ring rot diagnosis should not be based on symptomatology alone. Also, the use of a single confirmatory test in diagnosis may not be sufficient. The new semiselective agar medium, NCP-88, offers promise for improved detection of viable *C. m. sepedonicus* cells. When combined with serological and pathogenicity tests, the use of NCP-88 provides an improved technique for the detection of *C. m. sepedonicus* and diagnosis of ring rot.


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