APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1992, p. 740–743
0099-2240/92/020740-04$02.00/0

Vol. 58, No. 2

Selective Medium for Quantitation of *Bacillus popilliae* in Soil and in Commercial Spore Powders

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Received 13 June 1991/Accepted 26 November 1991

A medium consisting of MYPGP agar supplemented with vancomycin was found to be highly selective for *Bacillus popilliae*, especially for strains originally isolated from Japanese beetle larvae. The medium has proven to be useful for the quantitation of *B. popilliae* spores in commercial spore powder and in soil.

Bioinsecticidal powders containing spores of *Bacillus popilliae* and *Bacillus lentimorbus* have been used effectively for suppression of Japanese beetle (*Popillia japonica* Newman) populations (4, 8). Because of difficulties in obtaining sporulation in vitro (7, 14), one company (Fairfax Biological Laboratory, Inc., Clinton Corners, N.Y.) manufactures bioinsecticidal powder (Doom) by production of spores in vivo, i.e., in Japanese beetle larvae. At a late stage of the disease, referred to as milky disease, the number of spores in larval hemolymph may be as high as 5×10^{10}/ml (11). Doom consists of *B. popilliae* spores, derived from the macerated remains of infected larvae, plus inert material. Thus, Doom contains other spore-forming organisms in addition to *B. popilliae*. Thompson and Heimpel (17) determined that the average count of bacteria other than *B. popilliae* in five different batches of Doom was 6.1×10^4/g. To make quantitative checks on commercial spore products, it is desirable to have a selective medium which promotes growth of *B. popilliae* but not of the contaminating bacteria. A selective medium is also needed to quantitate the *B. popilliae* spore population in soil after application of spore powder.

Milner (9) developed a semiquantitative procedure for detection of *B. popilliae* subsp. *rhopaea* in soil. The procedure was based on the observation that spore germination of *B. popilliae* is very slow compared with that of most other sporeformers. Soil was suspended in a medium that promoted germination. The suspension was subjected to seven cycles of incubation and heating. Each cycle consisted of a 40-min incubation and then heating for 20 min at 70°C to kill germinated spores and vegetative cells. Further growth selectivity was provided by incubating the plated samples anaerobically (*B. popilliae* is facultative). This procedure is very time-consuming, and the possibility of quantitative error is great.

Pridham et al. (10) first observed that *B. popilliae* NRRL B-2309 and derivative mutant strains were vancomycin (Vm) resistant. Our study, based on this observation, reports the use of vancomycin in the development of a selective medium.

(A preliminary account of this work was presented at the 1991 General Meeting of the American Society for Microbiology [13].)

Table 1 lists *B. popilliae* and *B. lentimorbus* strains used in this study. Other *Bacillus* species used are listed in Table 3. Cultures were grown on MYPGP agar or in MYPGP broth (3) at 30°C.

Vancomycin resistance of *B. popilliae*, *B. lentimorbus*, and other *Bacillus* species. MICs of vancomycin were determined in culture tubes (16 by 125 mm) of MYPGP broth (5 ml) containing various concentrations of vancomycin. These tubes were shaken at a 45° angle in a rotary shaker for up to 4 days. Table 2 shows the lowest vancomycin concentration that prevented growth (the MIC) of several strains of *B. popilliae* and *B. lentimorbus*. These strains are grouped in Table 2 by their original insect host because host specificity might be correlated with vancomycin resistance. Variations in host specificity of strains may be evidence of existence of varieties or subspecies. (As an example of host specificity, *B. popilliae* strains isolated from Japanese beetles are notably lacking in their ability to infect *Cyclocephala* species [1, 7, 18].)

The strains exhibited either high resistance to vancomycin (growth at 100 μg/ml or more) or very low resistance (no growth at 1 μg/ml). Of the 16 strains of *B. popilliae* originally isolated from Japanese beetles, 15 were vancomycin resistant. This included the type strain, NRRL B-2309. All strains obtained from commercial spore preparations (*B. popilliae* Pj1, Pj2, and Pj5) were vancomycin resistant. Three strains of *B. lentimorbus* were tested; one was resistant to vancomycin and the other two were sensitive. All four of the *B. popilliae* strains originally isolated from oriental beetles were vancomycin resistant. In contrast, four of the *Cyclocephala* strains were vancomycin sensitive; only the *Cyclocephala hirta* strain was vancomycin resistant. Although patterns seem to be apparent, more strains isolated from insect hosts other than the Japanese beetle need to be tested before Vm^r-host specificity correlation can be established.

Table 3 shows the vancomycin MICs for several *Bacillus* species commonly found in soil. None of these species grew at vancomycin concentrations of >2.5 μg/ml. In a separate study (12), *B. polymyxa* and *B. amylolyticus* were found to be sensitive to 150 μg of vancomycin per ml. The MICs were not determined. The above results suggested that a vancomycin-supplemented medium might permit growth of resistant strains of *B. popilliae* while preventing growth of the more abundant *Bacillus* species found in soil.

Use of MYPGP-vancomycin agar to detect *B. popilliae* in commercial spore powder and in soil. Doom, specifically the

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TABLE 1. Strains of *B. popilliae* and *B. lentimorbus*

Bacterial strain[a]	Original host	Source
B. popilliae NRRL B-2309[b]	Japanese beetle, *Popillia japonica* Newman	L. Nakamura
B. popilliaea NRRL B-2519	Japanese beetle	L. Nakamura
B. popilliae NRRL B-2524	Japanese beetle	L. Nakamura
B. popilliae NRRL B-2527	Japanese beetle	L. Nakamura
B. popilliae Pj1	Japanese beetle; isolated from Milky Spore (Reuter Laboratory) purchased in 1980	D. Stahly
B. popilliae Pj2	Japanese beetle; isolated from Grub Attack (Reuter Laboratory; supplied to D. Potter, University of Kentucky, Mar. 1988; Reuter lot 2-83-736)	M. Klein and D. Stahly
B. popilliae Pj3	Japanese beetle; hemolymph slide (Wooster, Ohio; Oct. 1990)	M. Klein
B. popilliae Pj4	Japanese beetle; hemolymph slide (Moorestown, N.J.; 14 Mar. 1944)	M. Klein
B. popilliae Pj5	Japanese beetle; isolated from Doom (Fairfax Biological Laboratory, Inc.) purchased in 1983	M. Klein and D. Stahly
B. popilliae DNG2	Japanese beetle; hemolymph slide (Windsor, Conn.; 14 Apr. 1987)	J. Hanula and D. Dingman
B. popilliae DNG5	Japanese beetle; hemolymph slide (Groton, Conn.; 21 Apr. 1987)	J. Hanula and D. Dingman
B. popilliae DNG8	Japanese beetle; hemolymph slide (Norwalk, Conn.; 22 Apr. 1987)	J. Hanula and D. Dingman
B. popilliae DNG9	Japanese beetle; hemolymph slide (Danbury, Conn.; 18 May 1987)	J. Hanula and D. Dingman
B. popilliae DNG10	Japanese beetle; hemolymph slide (Hamden, Conn.; 6 Nov. 1990)	D. Dingman
B. popilliae KLN1	Japanese beetle; hemolymph slide (Wooster, Ohio; 15 May 1987)	M. Klein and D. Dingman
B. popilliae KLN3	Japanese beetle; hemolymph slide (Moorestown, N.J.; 18 Mar. 1945)	M. Klein and D. Dingman
B. lentimorbus Pj1	Japanese beetle; hemolymph slide (Akron, Ohio; 31 Jan. 1975)	M. Klein
B. lentimorbus KLN2	Japanese beetle; hemolymph slide (Akron, Ohio; 31 Jan. 1975)	M. Klein and D. Dingman
B. lentimorbus ATCC 14707	Japanese beetle (NRRL B-2522)	ATCC
B. popilliae DNG1	Oriental beetle, *Anomala orientalis* Waterhouse; hemolymph slide (Norwalk, Conn.; 22 Apr. 1987)	J. Hanula and D. Dingman
B. popilliae DNG4	Oriental beetle; hemolymph slide (Groton, Conn.; date uncertain)	J. Hanula and D. Dingman
B. popilliae DNG11	Oriental beetle; hemolymph slide (Groton, Conn.; 6 Nov. 1990)	J. Hanula and D. Dingman
B. popilliae DNG12	Oriental beetle; hemolymph slide (Groton, Conn.; 10 Oct. 1989)	J. Hanula and D. Dingman
B. popilliae Cp1	*Cyclocephala parallela* Casey (Belle Glade, Fla.); isolated from spore powder made in Mar. 1990	M. Klein and D. Stahly
B. popilliae DGB1	*Cyclocephala parallela*; hemolymph slide (South Fla.; 1986)	D. Boucias and D. Dingman
B. popilliae Cb1	Northern masked chafer *Cyclocephala borealis* Arrow (Ohio); isolated from spore powder made in 1983	M. Klein
B. popilliae Cb2	Northern masked chafer (Ohio); isolated from spore powder made in June 1985	M. Klein and D. Stahly
B. popilliae Ch1	*Cyclocephala hirta* LeConte (Moraga, Calif.); isolated from spore powder made in Nov. 1989	M. Klein and D. Stahly
B. popilliae Pa1	June beetle, *Phyllophaga anxia* (LeConte) (Todd, N.C.); isolated from spore powder made in Oct. 1984	M. Klein and D. Stahly
B. popilliae KLN4	*Anomala flavipennis* (Burmeister); hemolymph slide (Wilmington, N.C.; Sept. 1986)	M. Klein and D. Dingman

[a] These strains are available from D. P. Stahly and D. W. Dingman. NRRL, Northern Regional Research Laboratory, Peoria, Ill. For more information on the NRRL strains, see Gordon et al. (6). ATCC, American Type Culture Collection, Rockville, Md.

[b] The original source of *B. popilliae* NRRL B-2309, the type strain, is somewhat uncertain (6, 15, 16). It is possible that the original host was the European chafer, *Rhizotrogus majalis* (Razoumowsky).

batch from which *B. popilliae* Pj5 was isolated (Table 1), was used as the source of in vivo produced *B. popilliae* spores. Soil, air dried before use, was obtained from an area in Iowa City, Iowa. This area has not been exposed to commercial spore powder. Aqueous suspensions (5 ml) of Doom (1 g) and Doom plus soil (1 g each) were heated at 60°C for 15 min to kill most vegetative cells. Dilutions were made in H_2O, and 0.1-ml portions were plated on MYPGP agar and MYPGP agar plus vancomycin (150 μg/ml). Plates were incubated at 30°C for up to 2 weeks. Figure 1 shows an example of typical results. The colonies growing on MYPGP-plus-vancomycin agar were almost all *B. popilliae* on the basis of colonial and cellular morphology and a negative catalase reaction. *B. popilliae* colonies first appeared on this medium after about 5 days of incubation. Maximum colony counts were attained in about 9 days. *B. popilliae* spores in the particular batch of Doom analyzed were detectable without use of the selective medium, because the number of *B. popilliae* spores present was considerably higher than the number of spores of other *Bacillus* species. The number of *B. popilliae* spores detected on both selective and nonselective media was essentially the same: 4.1×10^8 spores per g on MYPGP agar and 4.7×10^8 spores per g on MYPGP-vancomycin agar. This result illustrates the fact that the colony-forming ability on the selective medium is comparable to that on the nonselective medium.

Soil suspensions cannot be diluted prior to plating when *B. popilliae* spores are present at very low concentrations in soil. Figure 1 shows that at low dilutions essentially confluent growth of bacteria other than *B. popilliae* occurs on MYPGP agar. Use of the selective medium allows detection in soil or commercial spore powder of *B. popilliae* spores present in concentrations as low as 5×10^3/g. The sensitivity would be greater, but only about 1% of *B. popilliae* spores visible by phase-contrast microscopy germinate and grow to produce colonies on MYPGP agar (2). Occasionally, mold growth becomes excessive during the long incubation period required for development of *B. popilliae* colonies. Mold growth can be suppressed by incorporation of 1.0 g of

TABLE 2. Vancomycin resistance of various strains of *B. popilliae* and *B. lentimorbus*

Bacterial strain and original most	Lowest [vancomycin] preventing growth (μg/ml)	Highest [vancomycin] permitting growth (μg/ml)
Japanese beetle, *Popillia japonica* Newman		
B. popilliae NRRL B-2309	>1,000	1,000
B. popilliae NRRL B-2519	>1,000	1,000
B. popilliae NRRL B-2524	>1,000	1,000
B. popilliae NRRL-2527	>1,000	1,000
B. popilliae Pj1	1,000	500
B. popilliae Pj2	500	250
B. popilliae Pj3	1,000	500
B. popilliae Pj4	1,000	500
B. popilliae Pj5	1,000	500
B. popilliae DNG2	1,000	500
B. popilliae DNG5	500	250
B. popilliae DNG8	500	250
B. popilliae DNG9	1,000	500
B. popilliae DNG10	1.0	0.1
B. popilliae KLN1	1,000	500
B. popilliae KLN3	1,000	500
B. lentimorbus Pj1	1,000	500
B. lentimorbus KLN2	1.0	0.1
B. lentimorbus ATCC 14707	1.0	0.1
Oriental beetle, *Anomala orientalis* Waterhouse		
B. popilliae DNG1	1,000	500
B. popilliae DNG4	250	100
B. popilliae DNG11	500	250
B. popilliae DNG12	500	250
Cyclocephala parallela Casey		
B. popilliae Cp1	1.0	0.1
B. popilliae DGB1	1.0	0.1
Northern masked chafer, *Cyclocephala borealis* Arrow		
B. popilliae Cb1	1.0	0.1
B. popilliae Cb2	1.0	0.1
Cyclocephala hirta LeConte		
B. popilliae Ch1	>1,000	1,000
June beetle, *Phyllophaga anxia* (LeConte)		
B. popilliae Pa1	1.0	0.1
Anomala flavipennis (Burmeister)		
B. popilliae KLN4	1,000	500

TABLE 3. Vancomycin resistance of *Bacillus* species other than *B. popilliae* and *B. lentimorbus*

Strain	Source[a]	Lowest [vancomycin] preventing growth (μg/ml)	Highest [vancomycin] permitting growth (μg/ml)
B. cereus T	H. Halvorson	2.5	1
B. megaterium 899	G. Starka	1	0.1
B. subtilis 168	J. Ito	1	0.1
B. larvae NRRL B-3555	L. Nakamura	2.5	1
B. brevis ATCC 8246	ATCC	2.5	1
B. licheniformis A-5	R. Bernlohr	1	0.1
B. pumilus ATCC 7061	ATCC	1	0.1
B. sphaericus 7582	D. Tipper	1	0.1

[a] ATCC, American Type Culture Collection.

cycloheximide per liter into the MYPGP-plus-vancomycin agar (see reference 5).

Use of 150 μg of vancomycin per ml in the selective medium may be excessive. In one experiment in which a heated soil suspension was plated, growth of *Bacillus* species other than *B. popilliae* was inhibited equally as well by 10 μg as by 150 μg of vancomycin per ml.

At present, commercial spore powders produced in the United States are made specifically for control of the Japanese beetle. The selective medium developed in this study should be useful in future quantitative studies of Japanese beetle-specific *B. popilliae* strains.

Interest in developing commercial preparations to control *Cyclocephala* populations is increasing (7, 8, 18). In our limited survey of *B. popilliae* strains originally isolated from *Cyclocephala* hosts, we found that only one of five strains is resistant to vancomycin. In this case, the selective medium may not be as generally applicable as it is for the Japanese beetle strains. If commercial *Cyclocephala* strains which are vancomycin resistant are used (e.g., *B. popilliae* Ch1), then this selective medium will be applicable. Alternatively, in the future, it may be possible to genetically transfer vancomycin resistance to vancomycin-sensitive strains.

We gratefully acknowledge the provision of *B. popilliae* and *B. lentimorbus* hemolymph slides and commercial spore preparations by M. Klein and J. Hanula. Thanks to both also for helpful

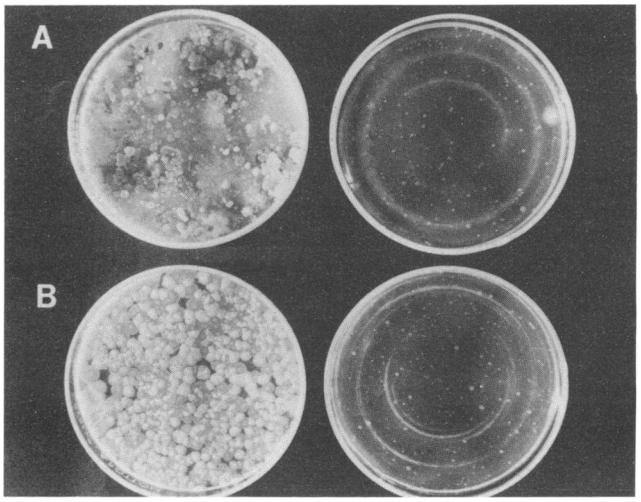

FIG. 1. Growth of bacteria on MYPGP agar and on MYPGP agar plus vancomycin. (A) Doom plus soil on MYPGP (left) and on MYPGP plus 150 μg of vancomycin per ml (right). (B) Doom on MYPGP (left) and on MYPGP plus 150 μg of vancomycin per ml (right). Prior to plating, aqueous suspensions were prepared and heated as described in the text. The results represent plating of 0.1-ml portions of a 10^{-2} dilution (A) and a 10^{-1} dilution (B). Plates were incubated at 30°C for 2 weeks.

discussions during the course of this investigation. We also thank Linda Riggan for secretarial assistance.

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