APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1995, p. 1839–1842 Vol. 61, No. 5
0099-2240/95/$04.00+0

A Soil and Rhizosphere Microorganism Isolation and Enumeration Medium That Inhibits *Bacillus mycoides*

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Received 26 August 1994/Accepted 9 March 1995

A new solid medium has been developed for the enumeration and isolation of soil and rhizosphere microorganisms. This medium, named rhizosphere isolation medium, contains glucose and 15 of the 20 common amino acids. The absence of five other amino acids, namely, aspartic acid, asparagine, cysteine, proline, and threonine, inhibits the growth of *Bacillus mycoides*, a commonly encountered bacterium that rapidly spreads on agar media and complicates the isolation and enumeration of other microorganisms. Compared with a similar medium containing Casamino Acids, rhizosphere isolation medium had half as many colonies of *B. mycoides*, with each colony approximately half the diameter. The two media had similar total numbers of bacterial colonies. Isolates were divided into taxononomic groups, roughly corresponding to species and genus, by fatty acid methyl ester analysis and numerical methods. There were 24 genera and 41 species found in the isolates from rhizosphere isolation medium, while 19 genera and 35 species were found in the isolates from the medium prepared with Casamino Acids. No major group of bacteria was found to occur only on one medium or on the other, indicating that the five missing amino acids had no great effect on organisms other than *B. mycoides*. This medium may prove useful in soil and rhizosphere studies in which the growth of *B. mycoides* is undesirable.

Numerous selective and nonselective media have been used for enumerating and isolating soil and rhizosphere microorganisms. It is clear that the choice of medium has a great impact both on the total count and on the relative abundances of various taxonomic groups of microorganisms. Fluorescent pseudomonads have been reported to be a dominant group in the rhizosphere, but this may be an artifact of using rich media and high temperatures, conditions under which fast-growing heterotrophs can dominate (4). The populations of soil bacteria isolated on three different media were found to be significantly different from each other (6). When soil bacteria were plated out on various dilutions of nutrient broth solidified with agar, numbers were greater on nutrient broth diluted 100-fold than on full-strength nutrient broth, and many isolates were found to grow only on dilute formulations (8). A dilute medium was found to yield higher numbers of fluorescent pseudomonads than the same medium at a higher concentration (7). It therefore appears that a dilute medium might better serve as a nonselective agar for counting and isolation purposes.

For certain areas of research, such as physiological studies and screening for biological control organisms, it is desirable to use a growth medium with a chemical composition similar to that of the natural habitat of the microorganisms in question. We previously reported on rhizosphere medium (RSM) (2), a rich medium containing Casamino Acids and sucrose, with inorganic constituents based on the ionic composition of a typical soil solution. We subsequently used a variant of RSM, with no Casamino Acids and alternative carbon sources for sucrose, to study the effect of carbon source on antibiosis between a bacterium and a fungus (1). A dilute variant of RSM, containing low levels of organic carbon and nitrogen, may serve as a relatively nonspecific isolation medium.

Bacillus mycoides, a common soil microorganism, shows a rhizoid spreading growth on solid media (5). The rhizoid filaments interfere with certain automated plate counting devices and also make it difficult to obtain pure cultures of other organisms. We report here on the development of a diluted form of RSM with 15 amino acids which inhibits the growth of *B. mycoides* and is relatively nonselective for other bacteria.

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MATERIALS AND METHODS

Media. All chemicals were reagent grade unless otherwise noted. Rhizosphere isolation medium (RIM) was prepared as follows. All stock solutions are given in Table 1. A total of 10.0 ml of 100× $Ca(NO_3)_2$, 1.0 ml of 1 M $MgSO_4$, 9.11 g of ACES (*N*-[2-acetamido]-2-amino-ethanesulfonic acid), and 1.0 g of NaOH were dissolved in 500 ml of H_2O. After confirming that the pH was 6.7 to 6.9, 15 g of agar (BBL Agar Granulated) was added. The mixture was brought to 1,000 ml with H_2O and autoclaved. After cooling to 45 to 50°C, the following stock solutions were added: 1.0 ml of 1 M KH_2PO_4, 1.0 ml of glucose, 1.0 ml of amino acid mixture, 1.0 ml of tyrosine, 1.0 ml of vitamins, 20.0 ml of cycloheximide, and 5.0 ml of nystatin. Plates were poured, allowed to sit overnight at room temperature, and stored at 4°C.

Dilute RSM (dRSM) was prepared identically, except that sucrose was used instead of glucose and 1.0 ml of 10% Difco Bacto-Casamino Acids was used instead of the amino acid mixture and tyrosine. Trypticase soy broth agar contained 30 g of Trypticase soy broth (BBL) and 15 g of granulated agar (BBL) per liter. ACES-buffered saline contained 8.5 g of NaCl and 3.6 g of ACES per liter and was adjusted to pH 6.7 to 6.9 with NaOH before autoclaving.

Strains. The following strains of *B. mycoides* were obtained from the American Type Culture Collection (Rockville, Md.): 6462^T (type strain), 23258, and 31102. *B. mycoides* was also obtained from Sassafras sandy loam (Typic Halpdults) soil taken from a field at the Beltsville Agricultural Research Center, Beltsville, Md., by plating soil extracts on dRSM. *B. mycoides* was isolated by the characteristic spiral pattern of the rhizoid colonies (5) and identified by fatty acid methyl ester analysis, according to the standard protocols provided by Microbial ID (MIDI, Newark, Del.). *B. mycoides* was maintained on dRSM and on Trypticase soy broth agar.

Extracts. Bacteria were extracted from soil and rhizosphere samples by placing approximately 10 g of soil or roots plus adhering soil into a sterile, tared 250-ml Erlenmeyer flask sealed with a cotton plug. The flask was weighed, and the sample weight was calculated. A volume of ACES-buffered saline equivalent to nine times the sample weight was added, and the flask was shaken at 200 rpm for 10 min. The slurry was serially diluted 10-fold in sterile ACES-buffered saline.

Enumerations. The diluted extracts were plated in duplicate with a Spiral Plater Model D (Spiral Biotech, Rockville, Md.). After incubation at 22°C for 2 to 3 days, the colonies were counted either manually or with a Model 500A Laser Colony Scanner with BEN software (Spiral Biotech).

Identification of bacteria. Single colonies were streaked on Trypticase soy

TABLE 1. Stock solutions used in the preparation of RIM

Stock	Ingredient	Concn (g liter^{-1})	Diluent	Treatment
100× $Ca(NO_3)_2$	$Ca(NO_3)_2 \cdot 4H_2O$	75.1	H_2O	Autoclave
1 M $MgSO_4$	$MgSO_4 \cdot 7H_2O$	246.5	H_2O	Autoclave
1 M KH_2PO_4	KH_2PO_4	136.1	H_2O	pH 7 with NaOH, autoclaving
Glucose	Glucose	100.0	H_2O	Autoclave
Amino acid mixture	Gly, Ala, Val, Leu, Ile, Ser, Met, Phe, Trp, Glu, Gln, His, Arg, Lys	6.7 of each amino acid	H_2O	Autoclave
Tyrosine	Tyr	6.7	H_2O	NaOH to dissolve, autoclaving
Vitamin	Biotin	0.10	H_2O	Filter sterilization
	Niacinamide	0.35		
	Thiamine · 2HCl	0.30		
Cycloheximide	Cycloheximide	20.0	H_2O	Filter sterilization
Nystatin	Nystatin	10.0	Methanol	

broth agar, incubated at 28°C, and restreaked if necessary to ensure purity. Bacteria were identified by fatty acid methyl ester analysis according to the standard protocols provided by MIDI. Cluster analysis was accomplished by the single-link method with Dendrogram II (MIDI). Principal component analysis was performed with NTSYS-pc (Exeter Software, Setauket, N.Y.).

RESULTS AND DISCUSSION

When soil extracts were plated out on dRSM, colonies of *B. mycoides* spread across the entire plate in 3 to 5 days. This made the colonies difficult to count manually and impossible to count with the Laser Colony Scanner. The *B. mycoides* contaminated many other colonies on the plates so that several restreakings were required to obtain single colonies of other bacteria. When soil extracts were plated out on a variant of RSM, containing no Casamino Acids, no *B. mycoides* colonies were found. This suggested that *B. mycoides* growth required or was stimulated by some amino acids.

A series of experiments were performed to determine if any particular amino acids were essential or stimulated growth of *B. mycoides*. Four different solid media, based on RSM without Casamino Acids, were prepared, and each contained 5 of the common 20 amino acids. One medium (A), containing tryptophan, cysteine, proline, aspartic acid, and asparagine, yielded the best growth of *B. mycoides*, while another medium (B) containing serine, methionine, threonine, phenylalanine, and tyrosine, supported some growth. Two other media, one (C) containing glycine, alanine, valine, leucine, and isoleucine, and another (D) containing glutamic acid, glutamine, histidine, arginine, and lysine, supported very little growth of *B. mycoides*. The 10 amino acids from the latter two media (C plus D) were combined in another solid medium and still yielded little growth of *B. mycoides*. Finally, various combinations of amino acids from the first two media on which *B. mycoides* grew well (A and B) were added to the 10 amino acids that did not stimulate growth (C plus D) in an effort to find a medium, containing as many of the common amino acids as possible, on which *B. mycoides* would not grow well.

We determined that aspartic acid, asparagine, cysteine, proline, and threonine all stimulated the growth of *B. mycoides*. We prepared RIM which contains 15 of the 20 common amino acids, eliminating those amino acids found to stimulate the growth of *B. mycoides*. Nystatin and cycloheximide were added to inhibit fungi and actinomycetes.

This medium was tested first with the three ATCC strains of *B. mycoides*. Strains ATCC 6462^T and 23258 grew much more rapidly at 22°C on dRSM than on RIM. Growth of *B. mycoides* (ATCC 31102) was inconsistent on both media. Some colonies on some plates showed rapid growth, and some plates showed no growth at all.

To further test the medium, extracts were then prepared from fresh soil samples taken from two different fields at Beltsville, one consisting of Sassafras loam and the other consisting of Christiana silt loam, and plated out on both media. After 1 day of incubation, colony counts were significantly higher on dRSM, but after 2 days, no significant differences were observed. After 1, 2, and 3 days, the *B. mycoides* growth was much less on RIM than on dRSM. At 3 days, the colonies of *B. mycoides* were approximately one-half as large on RIM as on dRSM.

For the final test, barley plants were grown in the same two soils as well as Comly loam from a field at the Rodale Institute Research Center, Kutztown, Pa. After 2 weeks of growth, rhizosphere samples were extracted and plated on RIM and dRSM. Colonies were counted after 2 days. There were no significant differences between the counts obtained on the two media (Table 2). There were approximately half as many *B. mycoides* colonies on RIM as on dRSM, and colony diameters averaged 3 to 5 mm for RIM and 5 to 10 mm for dRSM, as shown in Fig. 1.

It seemed probable that bacteria other than *B. mycoides* might also require one or more of the five amino acids omitted from RIM. To test this possibility, randomly selected colonies were taken from the plates prepared from the Comly loam and subjected to fatty acid methyl ester analysis. A total of 329 isolates from six plants were analyzed. Of these, 164 were from the RIM plates while 165 were from the dRSM plates. On both media, three species were found to be the most abundant and were identified by fatty acid methyl ester composition as *Bacillus megaterium*, *Bacillus maroccanus*, and an *Arthrobacter* species. In order to avoid the problem of strains not matching the library provided, dendrograms were constructed and used

TABLE 2. Bacterial numbers in barley rhizosphere soils from three different sites[a]

Site	RIM		dRSM	
	Mean ($\log_{10}$ CFU g^{-1})	SD	Mean ($\log_{10}$ CFU g^{-1})	SD
Rodale	6.20	0.10	6.27	0.14
Beltsville Christiana	6.50	0.09	6.59	0.14
Beltsville Sassafras	6.86	0.27	6.79	0.23

[a] All results are on a fresh weight basis. Each result is the mean of six replicates.

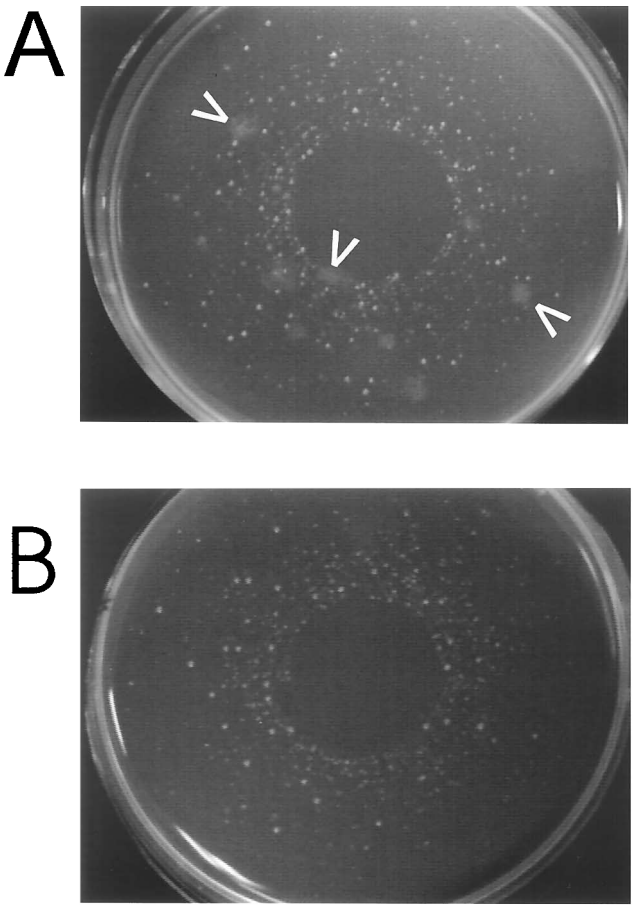

FIG. 1. Comparison of bacterial growth on dRSM and RIM. Rhizosphere extracts from plants grown in soil from a Beltsville field were diluted 100-fold, plated, and incubated at 22°C for 2 days. (A) dRSM; (B) RIM. *B. mycoides* appears as large white colonies with poorly defined edges in panel A, and three *B. mycoides* colonies are marked with arrowheads.

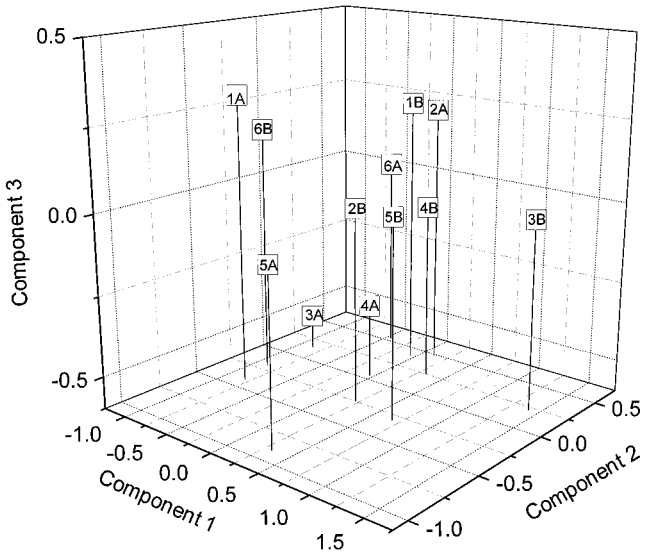

FIG. 2. Principal component analysis of rhizosphere "species" distribution from six plants as determined on two different media. (A) RIM; (B) dRSM.

to assign organisms to taxa at two hierarchical levels. Isolates linked at a Euclidean distance of five or less were considered to belong to the same "species" while linkages of five to eight were considered to indicate the same "genus." These values were based on dendrograms constructed from the MIDI library entries for various taxa and, while somewhat arbitrary, roughly correspond to standard taxonomic groupings. There were a total of 36 genera among the 329 isolates, with 24 genera among the RIM isolates and 19 genera among the dRSM isolates. Two hundred ninety-eight isolates were classified into seven genera that were found in both the RIM and dRSM groups. Nineteen isolates in 17 genera were found only in the RIM group, while 12 isolates in 12 genera were found only in the dRSM group. There were a total of 63 species, with 41 species found among the RIM isolates and 35 species represented among the dRSM isolates. Two hundred seventy-five isolates were classified into 13 species that were found in both the RIM and the dRSM groups. Thirty-three isolates in 28 species were found only in the RIM group, and 23 isolates in 22 species were found only in the dRSM group.

Since 91% of the isolates, when classified at the genus level, and 84% of the isolates, when classified at the species level, were found in taxa that occurred in both media, we concluded that the medium used did not affect the dominant bacteria. This was also demonstrated by the fact that no more than three isolates were found in any species or genus found in only one medium. Finally, analysis of variance was conducted on the number of isolates from each plant in each taxon, and no statistically significant ($\alpha = 0.05$) differences were found.

Principal component analysis of the species distribution was used to compare the samples. In this analysis, each sample, consisting of the population of bacteria from one plant on a given medium, was defined by the number of isolates in each species. Each sample could then be represented as a point in n-dimensional space, where n is the total number of species for all of the samples. Principal component analysis projects the n-dimensional data onto three orthogonal coordinate axes (three-dimensional space) while maximizing variance between samples. As shown in Fig. 2, the samples did not cluster according to the medium used. This indicated that, for the 164 to 165 isolates from each medium, the medium used did not support the growth of a significantly different set of isolates.

It is possible that if many more plants and isolates were analyzed, differences would be detectable between the microbial populations found on the two media. However, it is clear from this study that any such differences must be quite small. There were few, if any, bacteria in this study that required the five amino acids missing from RIM. Of course, it is possible that certain rhizosphere bacteria do not grow on either of these media. However, no single medium can supply all the requirements for all bacteria (3), and evidence (4) shows that more-dilute media produce a more representative sample of the naturally occurring rhizosphere and soil bacteria than richer media.

RIM was designed to specifically inhibit the growth of *B. mycoides*. Undoubtedly, there are strain-to-strain differences in *B. mycoides*, and there are probably strains which will not be inhibited by this medium. However, two ATCC strains and isolates from three different field soils were inhibited, thus indicating that this medium has a potential advantage as an enumeration and isolation medium for soil and rhizosphere bacteria.

ACKNOWLEDGMENTS

I thank Stan Tesch for technical assistance and Patricia Millner for many helpful discussions and for review of the manuscript. I also thank Thierry Heulin and Jeff Fuhrmann for their critical reviews of the manuscript.

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