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A New Solid Medium for Enumerating Cellulose-Utilizing Bacteria in Soil

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A solid medium containing ashed, acid-washed cellulose and a dye, Congo red, has been developed for enumeration of cellulose-utilizing bacteria in soil. Bacteria able to use the cellulose in this medium produced distinct zones of clearing around their colonies. A vivid contrast between the uniform red color of the medium and these halos made this method of differentiation of these organisms superior to other methods.

Terrestrial plants contribute $>70 \times 10^9$ Mg of carbon annually to the global carbon budget (20). Consequently, soil microorganisms that catabolize the cellulosic material of plants (40 to 60% of plant residues) influence the flow of energy from plant material to higher trophic levels and ultimately the release of CO_2 to the atmosphere (1, 10, 20, 22). As soil bacteria and fungi are the microorganisms largely responsible for the turnover of plant material, changes in the numbers of these microorganisms can be related to changes in the organic matter content of soil (19, 33). When corroborated with other ecological indicators (e.g., biomass and species diversity measurements), changes in the numbers of these organisms can also provide an indication of changes in soil health and productivity (12, 18). Consequently, precise methods for enumeration of these microorganisms in soil are needed.

Several different approaches for the selective enumeration of cellulose-utilizing bacteria in soil have been described (for examples, see references 15, 24–26, and 31). The common basis of most methods is the hydrolysis of a cellulosic substrate. Liquid media are available for qualitative and quantitative estimates of cellulose-utilizing bacteria (for an example, see reference 16), but solid media are generally preferred. For example, Hankin and Anagnostakis (8) looked for zones of clearing surrounding colonies growing on agar containing cellulose. Others have flooded agar plates containing carboxymethylcellulose with a solution of Congo red to enhance the detection of bacterial colonies able to use cellulose (32). Current methods used to enumerate cellulosic bacteria in soil are often time-consuming. They may require strong acid for preparing specific cellulosic substrates. Furthermore, the colonies of cellulose-utilizing bacteria are often difficult to differentiate from other organisms on solid media (9, 25, 28, 31), even with polysaccharide precipitants, e.g., hexadecyltrimethylammonium bromide (HAB) (4).

Teather and Wood (29) and Wood et al. (32) observed that Congo red was useful in the assay of polysaccharide hydrolysates, showing that the dye formed complexes with unhydrolyzed polysaccharides. On the basis of that observation and those of others (7, 13, 17), we developed a new solid medium that contained Congo red, Noble agar, and gelatin. This new medium facilitated visual differentiation between bacteria that use cellulose and those that do not. On this medium, colonies surrounded by zones of clearing contrasted vividly with those that did not use the cellulose.

Three separate media were prepared in order to assess their abilities to recover and differentiate cellulose-utilizing bacteria from soil: (i) cellulose-Congo red agar, (ii) gelatin agar, and (iii) Sigmacell agar (Table 1). The cellulose-Congo red agar consisted of 0.50 g of K_2HPO_4 (Fisher Scientific, San Francisco, Calif.), 0.25 g of $MgSO_4$ (Sigma Chemical Co., St. Louis, Mo.), 1.88 g of ashed, acid-washed cellulose powder (J. T. Baker Chemical, Inc., Phillipsburg, N.J.), 0.20 g of Congo red (J. T. Baker), 5.00 g of Noble agar (Difco Laboratories, Detroit, Mich.), 2.00 g of gelatin (Difco), 100 ml of soil extract (31), and 900 ml of tap water (used to provide essential trace elements for soil bacteria). The gelatin agar contained all of the above ingredients except the Congo red dye. A 20-μm microcrystalline form of cellulose (Sigmacell type 20; Sigma Chemical Co.) was used instead of the ashed, acid-washed cellulose powder in the Sigmacell agar. Furthermore, this medium did not contain Congo red, Noble agar, or gelatin. Twenty grams of Bacto Agar (Difco) was used instead of Noble agar and gelatin (28). The cellulose-Congo red and gelatin agars were prepared by mixture of all of the ingredients, except the Noble agar and gelatin, in 900 ml of tap water. After adjustment of the pH to 7.0 with 1.0 N NaOH (J. T. Baker), the Noble agar and gelatin were dissolved in the medium by heating on a magnetic stirrer-hot plate. The medium was then autoclaved for 20 min at 121°C, cooled, dispensed into sterile petri dishes, air dried in a sterile hood, and stored at 4°C. For soil isolates, filter-sterilized cycloheximide (100-μg/ml final concentration; Sigma Chemical Co.) was added to the medium after autoclaving to prevent fungal growth.

To test the effectiveness of the new medium, the growth of known cellulose-utilizing bacteria (*Streptomyces lividans* TK23 [3, 10, 20]), non-cellulose-utilizing bacteria (*Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas putida* [11]), and cellulose-utilizing fungi (*Acremonium* sp. and *Alternaria* sp. [21]) was examined. Cultures of *Acremonium* sp. and *Alternaria* sp. were maintained on potato dextrose agar; *S. lividans* TK23 was maintained on yeast-malt extract agar (5); and *E. coli*, *P. mirabilis*, and *P. putida* were maintained on Luria-Bertani agar (Difco). Bacterial cultures (positive and negative controls) were grown in liquid culture for 18 h at 30 ± 2°C (mean ± range), serially diluted by decades, spread plated on the cellulose-Congo red agar, and incubated for 7 days at 25 ± 2°C. Following incubation, the colonies exhibiting zones of clearing

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TABLE 1. Numbers of cellulose-utilizing bacteria in three different soils as determined with three different media

Soils	Log_{10} no. of cellulose-utilizing bacteria/g of soil[a] (mean, SEM)[b]					
	Soil extract agar[c]	Cellulose-Congo red agar	Gelatin agar[d]		Sigmacell agar[e]	
			Agar alone	Agar + HAB	Agar alone	Agar + HAB
Thatuna-Naff	5.98, 0.04	4.90, 0.03	4.90, 0.05	4.95, 0.05	5.60, 0.03	5.77, 0.03
Waldo	6.38, 0.02	5.43, 0.02	5.84, 0.03	5.94, 0.03	5.90, 0.03	6.09, 0.02
Xeric-aridic	5.78, 0.04	4.60, 0.04	4.81, 0.02	5.23, 0.06	5.51, 0.05	5.65, 0.04

[a] Oven-dry equivalent.
[b] Each mean value represents nine replicates.
[c] Prepared from the indicated soil.
[d] Cellulose agar without Congo red.
[e] Prepared with Sigmacell type 20 cellulose.

were counted. Replicate plates of the bacterial cultures were flooded with a 1% solution of HAB (J. T. Baker) to examine the ability of HAB to enhance visualization of the zones of clearing (4). Small agar blocks of *Acremonium* and *Alternaria* cultures (additional positive controls) were placed directly on the surface of the cellulose-Congo red agar, incubated in the dark at 25 ± 2°C, and observed daily for zones of clearing. Furthermore, filter paper discs (7 mm in diameter) saturated with cellulase (ca. 0.18 U of cellulase per disc, from *Trichoderma viride*; Sigma Chemical Co.) were placed on cellulose-Congo red agar plates to determine whether cellulase alone could clear the medium.

The cellulose-Congo red agar was also evaluated by spread plate inoculation with 10-fold, sterile tap water dilutions of three soils of differing carbon content (14): (i) Thatuna-Naff silt-loam from western Idaho, (ii) Waldo silty clay loam from the Willamette Valley of Oregon, and (iii) xeric-aridic frigid soil from central Oregon. The physical and chemical characteristics of these soils are reported elsewhere (3, 6, 28). Dilution plates of soil extract agar were also inoculated to provide an estimate of the numbers of total bacteria in these soils (28, 31). The numbers of cellulose-utilizing bacteria detected by the different media were analyzed statistically with the general linear model for analysis of variance and Tukey's Studentized range test for differences between means (23, 27). The decision level for hypothesis testing was 0.05.

The three media tested were effective in supporting the growth of all organisms, and all of the bacterial and fungal strains tested grew on the cellulose-Congo red agar. Colonies of the positive controls (both bacterial and fungal) and the discs saturated with cellulase developed zones of clearing, but the negative controls did not (Fig. 1 and 2). The use of HAB to enhance the visualization of zones of clearing around colonies did not provide a substantial benefit in distinguishing cellulose-utilizing bacteria.

All three soils had distinct populations of bacteria with zones of clearing (Fig. 3; Table 1). When experienced personnel did the counting, the numbers of cellulose-utilizing bacteria detected in the three soils were significantly greater ($P = 0.05$) with the Sigmacell agar (with and without HAB) than with the other media (Table 1). Furthermore, the numbers of cellulose-utilizing bacteria detected with gelatin agar were significantly greater ($P = 0.05$) than with the cellulose-Congo red agar.

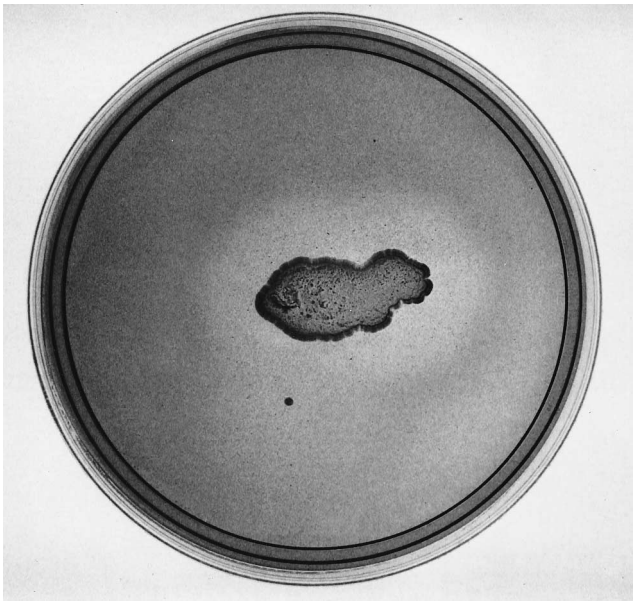

FIG. 1. A pure culture of *S. lividans* TK23, grown from a spore suspension, showing zones of clearing about the colonies. Certain species of *Streptomyces* are known to use cellulose as a carbon source.

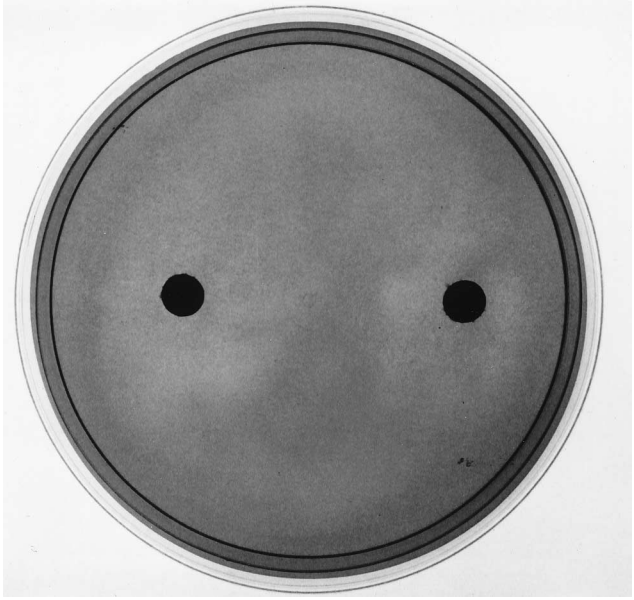

FIG. 2. Filter paper discs containing 0.18 U of cellulase (from *T. viride*). Note the diffuse zone of clearing about each disc. The extreme red color in the outer edges of the plate demonstrates the normal color depth of cellulose-Congo red agar.

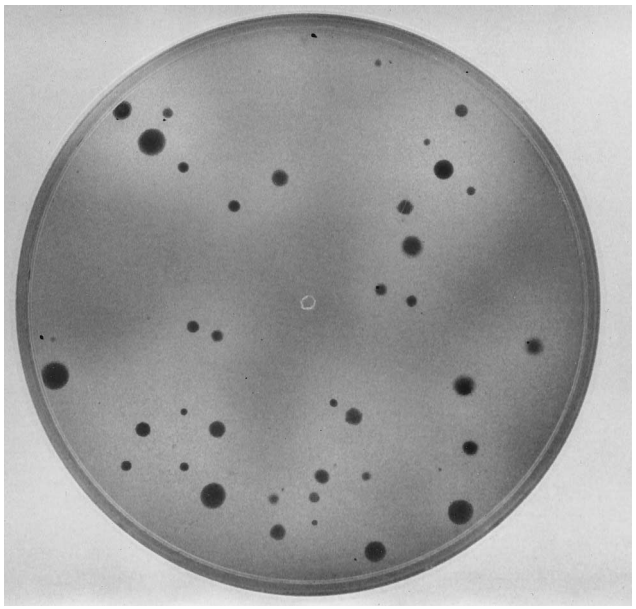

FIG. 3. Cellulose-utilizing bacteria derived from soil and grown on cellulose-Congo red agar. The colonies are red and surrounded by a zone of clearing.

These differences were not, however, exceptionally large. Moreover, colony differentiation on the gelatin and Sigmacell agars was quite variable (sometimes ≤50%; unpublished data) when inexperienced personnel did the counting. The underestimation of the numbers of cellulolytic organisms has also been reported by Bryant and Burkey (2). This underestimation of numbers might be improved, however, by careful control of the nature of the substrate (30).

The degree of variability in colony differentiation is perhaps the greatest factor affecting the reliability of media used to enumerate cellulose-utilizing bacteria in soil. The ease of distinguishing colonies with the cellulose-Congo red agar greatly reduced the subjectivity of deciding which colonies to count. Consequently, we rely on the cellulose-Congo red agar formulation in our present research.

Our speed and precision in enumerating cellulose-utilizing bacteria in soil were improved with the cellulose-Congo red agar. We found that (i) cellulose-utilizing bacteria were distinguishable on cellulose-Congo red agar by definitive zones of clearing around the colonies (generally red and, therefore, extremely easy to count); (ii) colonies of cellulose-utilizing bacteria could be enumerated on the cellulose-Congo red agar without the application of carbohydrate precipitants (e.g., HAB), eliminating any concern of cross-contamination; and (iii) cellulose-Congo red agar eliminated subjectivity in colony identification, enhancing the precision of the enumeration process. Work is presently underway to elevate the recovery efficiency of the cellulose-Congo red agar to that of the Sigmacell agar.

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