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Diffusion through a Double-Sided Plate: Development of a Method to Study Alga-Bacterium Interactions

FREDERICK S. COLWELL†* AND HAROLD K. SPEIDEL

Department of Biology, Northern Arizona University, Flagstaff, Arizona 86011

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Bacteria and algae isolated from a wastewater oxidation pond were inoculated onto opposing surfaces of double-layer agar plates (Lutri plates) to determine the usefulness of such plates for studying microbial interactions. The altered growth characteristics of various algae depending on the species of bacteria on the adjacent medium surface indicated that there was diffusion of extracellular products through the agar, suggesting that this simple assay can be used for screening potential interactions of actively growing organisms.

Recent studies involving the interactions of bacteria and algae stress the need for consideration of such microbial associations. Perhaps of greatest significance are the reports of coexistence of certain algae with the pathogen *Legionella pneumophila* and the apparent stimulation of bacterial growth under these conditions (3, 15, 17). Evidence of association of indicator bacteria with algae is also of interest in epidemiological studies (12). Positive chemotaxis of bacteria toward algae has also been demonstrated, and this suggests an association that is conducive to higher nitrogen-fixing rates in the algae (13). This interaction may be significant in promoting and maintaining algal blooms (14). In addition, there is ample evidence that bacteria often rely on extracellular products from algae as sources of organic carbon (1, 2, 9, 10, 20).

The studies described above indicate the need for methods of observing such interactions between organisms that exist in the same habitat. The use of solvent extracts of algal cultures in the preparation of test media has been described in several alga-bacterium association studies (5–7). While these investigations may facilitate the interpretation of microbial interactions, they depend on the extraction of cellular contents which are not normally excreted and therefore probably simulate algal cell lysis rather than secretion from intact cells. The use of filtrates from algal cultures is a suitable approach for studies involving relatively few isolates. In our study of the interaction of bacteria and algae commonly found in wastewater oxidation ponds, we were interested in the effects of many coexisting, viable organisms and therefore required a simple system which allowed the simultaneous analysis of interactions of such isolates.

Our objective was to test the utility of Lutri plates as a screening apparatus for qualitative interactions of bacteria and algae capable of growth on a solid agar surface. In addition, we were interested in the ability of wastewater pond microorganisms to enhance or inhibit the growth of one another by their diffusible extracellular products.

MATERIALS AND METHODS

Two operating wastewater oxidation ponds located in Williams and Clarkdale, Ariz., were selected for collection of bacteria and algae. Grab samples containing 200 ml of pond water were obtained throughout 1981 by using Whirlpak bags. Various stations at each site were sampled to increase the diversity of isolates. All samples were immediately transported to the laboratory and refrigerated within 3 h of collection.

Of the microbiological media used to isolate the bacterial components of the ponds, mENDO medium (Difco Laboratories, Detroit, Mich.) was selected to enhance the recovery of indicators of fecal pollution (in particular, *Escherichia coli*). Colonies of interest were screened by using the accepted scheme for total coliform bacteria (18). Both selenite F broth (Difco) and gram-negative broth (BBL Microbiology Systems, Cockeysville, Md.) selected for the growth of

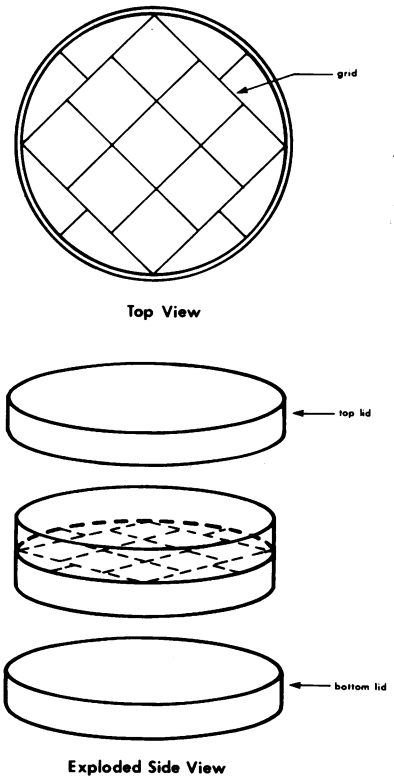

FIG. 1. Diagram of Lutri plate used to allow diffusion of extracellular products from organisms cultivated on opposing agar surfaces.

* Corresponding author.
† Present address: Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

TABLE 1. Algal growth associated with different species of bacteria plated on to the opposite surfaces of Lutri plates

Series	Alga		*Pseudomonas aeruginosa* isolate A	*Escherichia coli* isolate B	Coliform bacterium isolate C	Coliform bacterium isolate D	*Providentia* sp. isolate E	*Escherichia coli* isolate F	*Citrobacter freundii* isolate G	*Enterobacter cloacae* isolate H	*Pseudomonas* sp. isolate I	API group 1 isolate J[a]	*Hafnia alvei* isolate K	Control
	Taxon	Isolate												
1	*Chlorella vulgaris*	1	+[b]	+	+	+	+	+	+	+	+	+	+	+
	Ankistrodesmus	2	+	+	+	+	+	+	+	+	+	+	+	+
	Actinastrum	3	−	−	+/−	−	+/−	−	+/−	+/−	−	+/−	+/−	−
	Nitzschia	4	−	−	−	−	+	−	+	−	−	−	+	−
	Ulothrix	5	+	+	+	+	+	+	+	+	+	+	+	+
	Scenedesmus	6	+	+	+	+	+	+	+	+	+	+	+	+
2	*Scenedesmus*	7	+	−	−	−	+/−	−	−	−	+	−	+/−	+
	Nitzschia	8	+/−	+	+	+	+	+	+	+	+/−	+	+	+
	Nitzschia	9	−	+	+	+	+	+	+	+	+	+	+	+
	Chlorella	10	−	−	−	−	−	−	−	−	−	−	−	−
	Chlamydomonas	11	−	−	−	−	−	−	−	−	−	−	−	−
	Chlorococcum	12	+	+	+	+/−	+	+/−	+	+/−	+	+	+	+
3	*Chlorella*	13	−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−
	Scenedesmus	14	+	+	+	+	+	+	+	+	+	+	+	+
	Scenedesmus longus	15	+	+	+	+	+	+	+	+	+	+	+	+
	Phormidium	16	+/−	+	+	+	+	+	+	+	+	+	+	+
	Unidentified green alga	17	−	+	+	+	+	+	+	+	−	+	+	+
	Oscillatoria	18	−	−	+/−	−	−	+/−	−	−	−	−	−	−

[a] API group 1 is a special classification for organisms of indefinite identity (Analytab Products).
[b] +, Strong algal growth; −, no algal growth; +/−, weak algal growth.

intestinal pathogens potentially present in the ponds. Brilliant green, salmonella-shigella, and MacConkey agars (Difco) were used as selective and differential media for subculturing pond samples. Isolates were identified with API 20E strips (Analytab Products, Plainview, N.Y.).

Unialgal cultures were obtained by using the procedures described by Hoshaw and Rosowski (11) and Wiedeman et al. (19). Bold basal medium (4) was used either as a liquid medium or as a solid algal medium. This medium is essentially inorganic, except when agar is added, so that bacterial growth is minimized. Algal isolates were identified to genus by using *How to Know the Freshwater Algae* (16).

Lutri plates (diameter, 10 cm; Lutri Plate, Inc., Starkville, Miss.) allowed the cultivation of algae and bacteria on separate but adjoining solid medium surfaces (Fig. 1). Medium was first poured onto one surface, and, held by a removable plastic sheet, it solidified around a rigid grid in the center of the plate. Once the agar solidified, the plate was flipped, the sheet was removed, and a second layer of agar was poured on top of the lower surface of the first layer. Theoretically, stimulatory or inhibitory compounds from an organism growing on one medium could diffuse through the agar to affect an organism growing on the adjacent medium. These plates carried Bold basal medium on one surface and a synthetic sewage agar (8) on the opposing surface. The synthetic sewage agar contained a minimal concentration of salts, 0.5 g of glucose per liter, and 0.5 g of nutrient agar per liter.

The algal medium of each Lutri plate was inoculated peripherally with six unialgal cultures by using a Pasteur pipette. The same arrangement of algae was applied to 12 plates to make one series (Table 1). This provided 12 replicates of each series, 11 of which supported the growth of a different bacterial isolate on the synthetic sewage agar surface, thus allowing each alga and each bacterium an opportunity to interact. Standard petri plates with the same series of algae inoculated onto Bold basal medium were used as controls.

Algae were allowed to grow for 2 weeks at 25°C with constant illumination, and after this a single bacterial isolate was confluently swabbed onto the opposing synthetic sewage agar surface. The characteristics of growth of both algae and bacteria were observed 1 week after bacterial inoculation. The coloration and extent of colonial growth were compared with control and other treatment plates to determine the viability of algal and bacterial colonies under different conditions.

RESULTS AND DISCUSSION

The results of experiments in which Lutri plates were used to assay for agar diffusion of algal or bacterial products indicated that interactions were likely. Although this study was designed to show either adverse or beneficial effects on a bacterium according to which alga was plated opposite, in fact the results reflected changes in algal growth based on the bacterium which was cultivated on the opposing surface. Growth of the different bacterial isolates on the synthetic sewage agar side of each Lutri plate was apparent 1 week after inoculation. No inhibition or enhancement of the growth of these isolates was evident. Bacterial growth, although visible, was not prominent enough to photograph. The algae which were plated onto the Bold basal medium

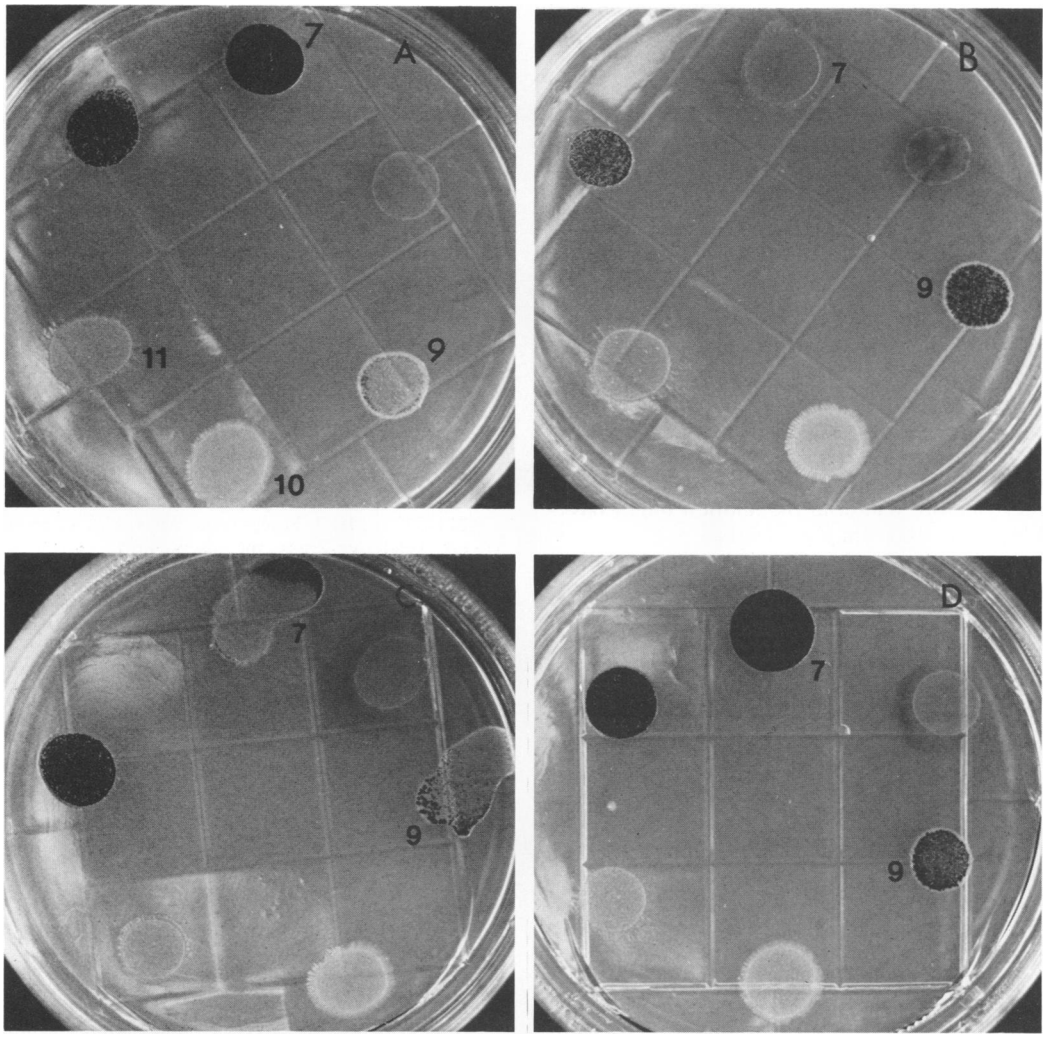

FIG. 2. Growth characteristics of *Scenedesmus* sp. isolate 7 and *Nitzschia* sp. isolate 9 when plated opposite *Pseudomonas aeruginosa* isolate A (A), *Enterobacter cloacae* isolate H (B), *Citrobacter freundii* isolate G (C), and *Pseudomonas* sp. isolate I (D) on Lutri plates.

surface varied considerably in degree and location of growth. In several situations this variability was clearly determined by the bacterial isolate present on the opposite surface of the Lutri plate.

Chlorella vulgaris isolate 1, *Ankistrodesmus* sp. isolate 2, *Scenedesmus* sp. isolates 6 and 14, and *Scenedesmus longus* isolate 15 all showed profuse growth after 3 weeks regardless of the bacterial isolate used (Table 1). *Ulothrix* sp. isolate 5 also grew well under all conditions; the only differences in growth may have been due to uneven inoculation of this filamentous alga. *Nitzschia* sp. isolate 8 appeared on each Lutri plate in series 2. However, its development was consistently weak. No growth was observed for *Chlorella* sp. isolate 10 or *Chlamydomonas* sp. isolate 11 on any of the plates in series 2. Most likely these algae simply did not flourish on Bold basal medium. *Actinastrum* sp. isolate 3 and *Nitzschia* sp. isolate 4, which were both present on series 1 plates, exhibited enhanced growth when they were plated opposite some bacteria. The overall appearance of these algae was often insufficient to draw conclusions.

Series 2 gave the most striking results. *Scenedesmus* sp. isolate 7 was apparently inhibited by all bacterial isolates with which it was combined except *Pseudomonas aeruginosa* isolate A and *Pseudomonas* sp. isolate I. Figure 2A shows evidence of profuse growth of *Scenedesmus* sp. isolate 7 when *Pseudomonas aeruginosa* isolate A was established on the opposite surface. In Fig. 2B, however, with *Enterobacter cloacae* isolate H on the opposite surface, this *Scenedesmus* sp. was completely suppressed. Figure 2C shows evidence of only partial inhibition of this alga by *Citrobacter freundii* isolate G, as shown by a separation of growing and nongrowing regions of the inoculum. Although not visible in the photograph, the edge of the swabbed area of *Citrobacter freundii* isolate G on the opposite surface was precisely aligned with the boundary of algal growth. This zonation graphically suggests that the presence of the bacterium had the effect of restricting the activity of the alga. Establishment of a diatom, *Nitzschia* sp. isolate 9, was inhibited by *Pseudomonas aeruginosa* isolate A (Fig. 2A). This suppression may be compared with the results shown in Fig. 2B and D, in which the same alga was capable of growth when it was exposed to different bacteria. Although *Pseudomonas aeruginosa* isolate A (Fig. 2A) and *Pseudomonas* sp. isolate I (Fig. 2D) each allowed growth of *Scenedesmus* sp. isolate 7, the effects which these bacteria had on the *Nitzschia* sp. were quite different.

In series 3, the two *Pseudomonas* isolates influenced the appearance of several of the algae tested (Table 1). *Chlorella* sp. isolate 13 grew weakly with most bacteria but was completely absent in the presence of *Pseudomonas aeruginosa* isolate A. *Phormidium* sp. isolate 16, a cyanobacterium, exhibited spreading growth on the agar surface except where it was inhibited by *Pseudomonas aeruginosa* isolate A. An unidentified green alga (isolate 17) survived poorly when it was combined with either of the *Pseudomonas* spp.

The most significant results are shown in Fig. 2; this figure shows that the growth of a single alga (*Scenedesmus* sp.) was modified according to the bacterium on the opposite surface. Before bacteria were inoculated onto the synthetic sewage agar, this *Scenedesmus* sp. exhibited no growth. After the establishment of two species of *Pseudomonas*, algal growth became apparent. Other bacteria either prevented growth of this alga or did nothing to reverse the conditions prior to their development. Lack of growth of this and other algae during the 2 weeks before bacterial inoculation might have been remedied by using different algal media.

Axenic cultures of algae would facilitate the interpretation of the results of the assay described above. Removing the influence of contaminating bacteria in nonaxenic cultures would simplify the assay by allowing interactions of only two organisms. Also, the examination of fewer organisms per plate is recommended. Crowding of algae on a single surface may lead to complicating interactions between algae.

Organic constituents of the synthetic sewage medium may have diffused through the agar to affect the algae on the opposite surface. This would be undesireable, but if heterotrophic growth is expected there is no apparent alternative. Although bacterial growth on the synthetic sewage medium was not profuse, probably due to nutrient limitation, algae may have been inhibited by the proximity of this organic medium. A medium with higher concentrations of organic compounds might lead to overgrowth of contaminating bacteria in nonaxenic algal cultures.

The limitations of this assay, which are similar to those of other procedures involving laboratory cultivation of microbes, suggest that the results obtained by using Lutri plates do not strictly imply interactions in the native system from which the microorganisms were isolated. Therefore, inferences concerning microbial interactions may be limited until further studies in which natural experimental conditions are used can be undertaken (e.g., parabiotic chambers or in situ studies).

The use of Lutri plates appears to be a promising qualitative method for screening alga-bacterium interactions. Our results indicate a striking contrast in algal growth responses when different bacteria are established on the adjacent surface of a Lutri plate. We believe that this technique is most applicable when the analysis for interactions involves numerous microorganisms of unknown character to be tested individually. The assay is simple both in theory and in practice and lends itself to photographic documentation. Results from preliminary studies with Lutri plates might lead to the selection of those species of greatest interest according to the goals of the investigator.

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