Application of a Rapid Method for Gram Differentiation of Plant Pathogenic and Saprophytic Bacteria Without Staining

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ABSTRACT

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The Gram-stain reactions of pathogenic and saprophytic bacteria isolated from plant parts were accurately characterized by mixing a concentrated droplet of cells with 3% potassium hydroxide (KOH). Compared to standard Gram-staining procedures, the KOH test was rapid, simple, and completely accurate. One hundred thirty strains representing *Achromobacter, Agrobacterium, Arthrobacter, Azotobacter, Bacillus, Corynebacterium, Erwinia, Pseudomonas, Xanthomonas,* and *Streptomyces* species were tested. Bacterial cells were aseptically removed from an agar medium with a toothpick and placed on a glass slide in a 50 μl drop of 3% KOH with rapid circular agitation. With Gram-negative strains the suspension became viscous as revealed by a mucoid thread that formed when the toothpick was lifted. Gram-positive bacteria dispersed into the drop and did not have this reaction. A few strains of *Corynebacterium* caused an increase in drop viscosity, but did not produce a mucoid thread.

Determination of positive or negative Gram-stain reaction is essential in the identification and classification of plant pathogenic bacteria. Schaad (6) stressed the importance of this test and reviewed some of the steps necessary to avoid inaccurate results. One problem often encountered when using Gram-staining techniques is that some Gram-positive bacteria will decolorize readily and some Gram-negative bacteria will have dark staining bodies depending on the culture medium and the stage of colony growth.

In 1938, Ryu (4,5) described a method for using potassium hydroxide (KOH) to differentiate Gram-positive and -negative bacteria without staining. This procedure greatly facilitated the identification of species being studied in experimental medicine. Gregersen (1) used this technique to accurately distinguish numerous Gram-positive and -negative strains including *Pseudomonas aeruginosa, P. cepacia, P. fluorescens, Streptomyces* spp., and *Bacillus* spp. often associated with plants. Halebian et al (2) have recently reported the successful application of the KOH test to differentiate clinical anaerobic bacteria.

This paper describes the application of Ryu's (4,5) technique for the separation of phytopathogenic and rhizosphere bacteria as to their Gram-stain reaction.

MATERIALS AND METHODS

Bacterial strains. Phytopathogenic and saprophytic bacteria used in these tests were obtained from the culture collection of the Department of Plant Pathology of the University of California at Berkeley or were from recent field isolations (see Table 1). Bacteria obtained from field isolations were identified by standard procedures (6). All bacteria were grown at 28 C for 24–48 hr (depending on growth rate) on King's medium B (3) for staining and KOH characterizations.

Staining and KOH tests. Gram-staining procedures utilizing ammonium oxalate-crystal violet, iodine solution, 95% ethyl alcohol as the decolorizing agent, and safranin O as the counterstain were followed as described (6).

Two drops, approximately 50 μl, of a 3% (w/v) solution of potassium hydroxide were placed on a clean glass slide as outlined by Ryu (5). Bacterial cells were transferred from culture media aseptically with a flat wooden toothpick and placed into the drop of KOH with rapid, circular agitation. After 5–8 sec, the toothpick was alternately raised and lowered just off the slide surface to detect a stringing effect (Fig. 1). The KOH test was considered positive if drop viscosity increased and stringing occurred within 15 sec.

RESULTS

The potassium hydroxide test for Gram-stain differentiation was in complete agreement with traditional Gram-staining procedures and accurately characterized 130 strains of phytopathogenic and rhizosphere-colonizing bacteria (Table 1). Gram-negative bacteria always caused an increase in KOH viscosity and formed a mucoid thread when lifted from the slide. In general, up to 7 days, age of the culture being tested did not affect the KOH test results. However,

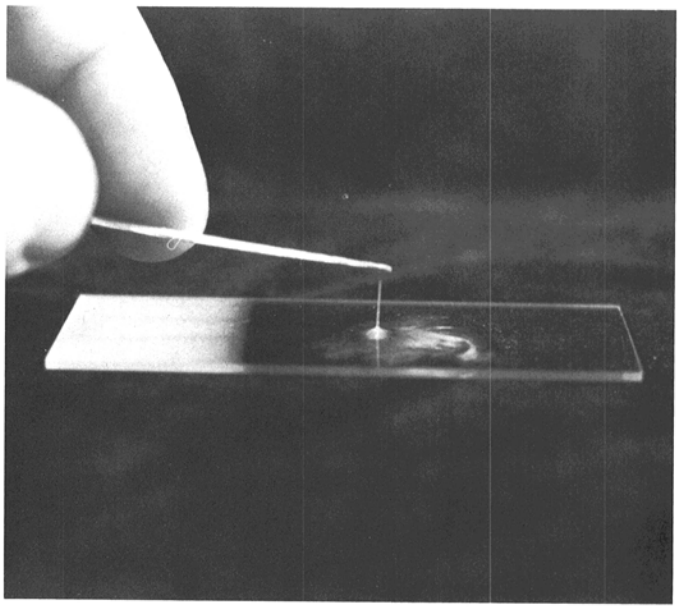

Fig. 1. Gram-negative bacterium giving positive mucoid threading in test with 3% KOH.

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TABLE 1. Comparison of Gram staining and Ryu's potassium hydroxide techniques for differentiation of strains of bacteria isolated from plants

Genus or species	Number of strains	Gram-stain reaction[a]	KOH reaction[b]
Achromobacter sp.	4	−	+
Agrobacterium radiobacter	3	−	+
A. tumefaciens	37	−	+
A. rhizogenes	1	−	+
Arthrobacter sp.	2	v	−
Azotobacter sp.	2	v	+
Bacillus cereus	2	+	−
B. megaterium	1	+	−
B. subtilis	3	+	−
Corynebacterium fascians	4	+,v	−
C. flaccumfaciens	3	+,v	−
C. michiganense	2	+	−
C. sepedonicum	5	+	−
Erwinia amylovora	2	−	+
E. carotovora			
subsp. *atroseptica*	4	−	+
subsp. *carotovora*	4	−	+
subsp. *betavasculorum*	4	−	+
E. chrysanthemi	4	−	+
Pseudomonas aeruginosa	2	−	+
P. cepacia	1	−	+
P. cichorii	1	−	+
P. fluorescens	4	−	+
P. marginalis	4	−	+
P. putida	3	−	+
P. syringae			
pv. *phaseolicola*	2	−	+
pv. *syringae*	6	−	+
pv. *tomato*	3	−	+
P. viridiflava	3	−	+
Xanthomonas campestris			
pv. *begoniae*	2	−	+
pv. *campestris*	2	−	+
pv. *incane*	1	−	+
pv. *juglandis*	3	−	+
pv. *malvacearum*	1	−	+
Streptomyces scabies	2	+	−
Streptomyces spp.	3	+	−

[a] Bacterial strains that retained a dark stain are designated as "+", those that decolorized are designated as "−". Strains that reacted with a variable or partial dark staining are designated by a "v."

[b] For KOH tests, "+" designates a mucoid stringing reaction and "−" designates absence of it.

some strains of *Agrobacterium* gave only a weak or no stringing reaction if cultures were older than 32 hr. Gram-positive bacteria had no reaction in KOH and generally dispersed into the drop. One isolate of *Cornyebacterium fascians* and one of *Corynebacterium flaccumfaciens* gave a slight increase in viscosity after 20 sec, but did not form a mucoid thread.

DISCUSSION

The use of the KOH test as an effective supplement to Gram staining has been very useful for the rapid and accurate differentiation of a large number of bacteria originally isolated from plant tissue. This method utilizes the rapid disruption of the cell wall of Gram-negative bacteria in alkaline solution releasing deoxyribonucleic acid which causes the viscous threading. Gram-positive bacteria do not lyse in 3% KOH. Just as Gregersen (1) found some exceptions with strains of *Bacillus*, we found that discrepancies or uncertainties could arise when testing *Agrobacterium* sp. if 24-hr cultures were not used. Nonetheless, Ryu's technique provides a supplementary diagnostic aid for plant pathologists. It must be remembered that a positive KOH reaction connotes a Gram-negative identity and vice versa.

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