FLAGELLA STAINING AS A ROUTINE TEST FOR BACTERIA[1]

H. J. CONN AND GLADYS E. WOLFE

New York State Agricultural Experiment Station, Geneva, N. Y.

Received for publication May 31, 1938

Nearly all descriptions of bacterial species contain statements as to whether or not motility is observed. The unsatisfactory character of this test has been realized for some time and various efforts have been made to improve upon the usual method, i.e., the microscopic examination of a hanging-drop. The most notable attempt of this sort in recent years is that by Tittsler and Sandholzer (1936), which is a macroscopic test depending upon the type of growth produced in a semi-solid agar.

In employing this semi-solid agar test on a series of soil organisms, certain plant pathogens, and organisms of the *Alcaligenes fecalis* and violet chromogenic groups, it was soon observed that, just as claimed by its originators, the test picks out many organisms that would not be recognized as motile by examination under the microscope. It has two disadvantages, however: it is rather slow, as cultures must be kept 3 or 4 days before a satisfactory reading can be made; it is uncertain, for considerable experience is necessary before it can be properly interpreted, and even an experienced user of the technic finds many tubes which give indefinite results. There proved, moreover, to be numerous cultures which appeared immotile in hanging-drop, and gave somewhat indefinite results by the semi-solid agar test, but which were shown on staining to have unmistakable flagella.

This raised the question as to whether it might be possible to devise a flagella-staining technic simple enough to be employed in routine pure culture study in the place of the conventional

[1] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 270.

test for motility. This has never been tried in the past because flagella staining methods are notoriously complicated and often fail to give identical results in the hands of different workers. Very recently, however, Hofer and Wilson (1938) have proposed a modification of the Gray flagella stain especially adapted for application to slime-forming bacteria. The results obtained with this technic were so consistent and were obtained with so little trouble, even in the hands of inexperienced technicians, that it seemed hopeful to modify the technic slightly so as to apply to other than slime-forming organisms. It was found easily possible to do this, the principal modification being the length of time to allow the organism to stand in water before making the smear on the slide. The procedure now employed in the case of miscellaneous bacteria is as follows:

PREPARATION OF GLASS SLIDES

Use new slides if possible. (This is because under the drastic method of cleaning to remove grease, old slides have a greater tendency to break, due to crystallization of the glass.) Clean first in a dichromate cleaning fluid, wash in water and rinse in 95 per cent alcohol; then wipe with a clean piece of cheese cloth. (Wiping is not always necessary but is advisable unless fresh alcohol is used after every few slides.) Pass each slide back and forth through a flame for some time, ordinarily until the appearance of an orange color in the flame; some experience is necessary before the proper amount of heating can be accurately judged.

Cool slides gradually in order to re-anneal, and thus to minimize breakage. A crude but ordinarily satisfactory method of annealing is to place the flamed slides on a metal plate (flamed side up) standing on a vessel of boiling water; and then to remove the flame under the water so as to allow gradual cooling. (Failure to anneal may result in breakage, sometimes as long as two weeks after the heating.)

PREPARATION OF SUSPENSIONS

Use young and actively growing cultures (e.g., 18–22 hours old), on agar slants. With a flamed *but well-cooled* loop transfer

a small amount of growth to 5–10 cc. of sterile distilled water, which has been held for several hours at room temperature. (Poor slides result from suspensions made in water that is too hot or too cold.)

Mix thoroughly in the distilled water and allow to stand 5–30 minutes, according to the type of growth produced. Gum-forming bacteria require 30 minutes, as recommended by Hofer and Wilson; those that produce no gum must stand in the water only 5–10 minutes. Standing in the water should be just long enough to allow the flagella to become untangled; too long a time results in their breaking off.

With a loop that has been flamed *and cooled*, remove a drop from the top of the suspension and place it on a glass slide prepared as above described. (The reason for taking material from near the surface of the suspension is because the non-motile cells tend to settle, while the motile cells—at least in the case of aerobes—collect at the top.) Smear the drop over the slide with the use of a second slide, as in preparing blood films; the film should be thin enough to dry rapidly and thus to minimize distortion.

STAINING

Use the mordant recommended by Gray (1926): 5 cc. saturated aqueous solution potassium alum; 2 cc. saturated aqueous solution mercuric chloride; 2 cc. 20 per cent aqueous solution tannic acid; 0.4 cc. saturated alcoholic solution basic fuchsin (presumably about 6 per cent, which was the strength employed in the present work). For best results, filter just before using. The technic is essentially that of Gray. Apply cold for 8–10 minutes; 10 minutes is ordinarily best, but this varies with the organism studied. (More than 10 minutes of mordanting is apt to cause too much precipitate.)

After mordanting, wash slides about 10 seconds in running water. Dry in the air, without heating. Stain 5 minutes, without heating, with Ziehl's carbol fuchsin; wash in running water; dry and examine. (In the present work a recently certified batch of National Aniline basic fuchsin was employed.)

In this procedure the most crucial points are the proper amount of heat to give the slides and the method of cooling so as to minimize the tendency to break. Although not all breakage can be avoided, the above-described method of cleaning the slides is recommended because of the better demonstration of flagella than when the preparations are made on slides cleaned by more ordinary procedures.

The technic thus developed proves simple enough so that it seems possible to recommend it as a routine test. It takes longer, to be sure, than the conventional hanging-drop test; but it gives a much higher percentage of positives. Results can be obtained much more quickly than by the semi-solid agar test of Tittsler and Sandholzer; they are more reliable, because interpretation of the results is never open to question; and finally, information is thus secured not only in regard to motility but also as to the type of flagellation. It is, in fact, hardly more difficult than the Gram stain, giving results that are more clear-cut and more easily interpreted.

REFERENCES

GRAY, P. H. H. 1929 A method of staining bacterial flagella. J. Bact., **12**, 273–274.

HOFER, A. W. AND WILSON, J. K. 1938 Use of the Gray flagella stain for slime-forming bacteria. Stain Techn., **13**, 75–76.

TITTSLER, RALPH P. AND SANDHOLZER, LESLIE A. 1936 The use of semi-solid agar for the detection of bacterial motility. J. Bact., **31**, 575–580.