Manual of
Microbiological Methods

H. J. CONN

MANUAL OF

Microbiological Methods

BY THE

SOCIETY OF AMERICAN BACTERIOLOGISTS

COMMITTEE ON BACTERIOLOGICAL TECHNIC

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McGRAW-HILL BOOK COMPANY, INC.

New York Toronto London

1957

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Library of Congress Catalog Card Number 57-8629

THE MAPLE PRESS COMPANY, YORK, PA.

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Preface

This manual is intended for use in those types of microbiological work which involve the study of microbial cultures or of viruses, either for identification or for learning the properties of the organisms investigated. This book takes the place of the loose-leaf publication issued during the period of 1923–1956 under the name of "Manual of Methods for Pure Culture Study of Bacteria." The present manual covers a wider scope but still includes the subject of "pure culture study," the meaning of which is discussed.

The methods given here are not to be regarded as official. The committee has always taken the stand that official methods should not be adopted in the case of research work, because it is continually necessary to modify research methods in order to keep them up to date. The standardization of methods tends to hinder the development of new technics, while the chief function of this committee is to stimulate its development.

The methods in this manual, therefore, are merely claimed to be those regarded as satisfactory by the committee at the time of publication. Whenever practical, the methods have been tested by the committee in comparison with other procedures.

Of the chapters in this book, V, VIII, IX, X, and XI are almost entirely new. The others are revisions, more or less complete, of leaflets of the old manual. In the case of these revised chapters, the names of the contributors at the chapter heads are the latest revisers, not the original authors. In the case of the five entirely new chapters, however, the actual authors are cited at the chapter heads.

H. J. Conn
M. J. Pelczar, Jr.

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CHAPTER I

Introductory

SCOPE OF THE MANUAL

There has sometimes been misunderstanding as to the sense in which the Committee on Bacteriological Technic uses the expression "pure culture study of bacteria." It is occasionally thought that such an expression would cover nearly the whole field of bacteriological technic. On the other hand, the definition of pure culture study of bacteria which has been drawn up by the committee is the study of bacterial cultures with the object of learning their characteristics and behavior or determining their identity, or both. Such a study may be regarded as including isolation methods, methods for the cultivation and the storage of various kinds of bacteria, microscopic study of pure cultures either stained or unstained, determination of cultural characteristics of an organism, a study of its physiological characteristics, the chemical methods necessary in making the last-mentioned study, the determination of pathogenicity and study of pathological effects, the serological characterisics of an organism when used as a means of description.

It is clear from such a statement that pure culture study of bacteria is fairly comprehensive but that there are many fields of bacteriological technic not included within it, e.g., methods for the enumeration of bacteria in their natural habitats, the diagnosis of disease and many other phases of medical bacteriology, methods employed in the study of food spoilage and controlling the processes of fermentation, etc. Such a list might be extended almost indefinitely, for the field of pure culture study, although fairly broad, is actually a small part, though basic, of bacteriological technic.

The scope of the present manual has been widened to include virological methods and procedures for the maintenance and preservation of bacteria, and it may in the future be expanded to include methods for yeasts and molds. Nevertheless its subject matter still does not cover those topics listed in the preceding paragraph.

RELATION TO TAXONOMY

Clearly, one of the objects of pure culture study is to determine the identity of any bacterial culture under investigation. This brings the

subject very close to the field of bacterial taxonomy, i.e., the naming and classifying of bacteria. Inasmuch as bacteria cannot be classified without studying their characteristics in pure culture, it is an obvious conclusion that pure culture study is a necessary prelude to bacterial taxonomy.

It must be recognized, nevertheless, that one can consider pure culture study without regard to taxonomy and that one can study the taxonomy of bacteria without paying special attention to the methods of pure culture study. Since this distinction can be made and the committee editing this series of publications is a committee on technic, care has always been taken to maintain the distinction so as not to interfere with the functions of other committees that have been appointed and to deal with matters of nomenclature and classification.

PUBLICATIONS OF THE COMMITTEE ON TECHNIC

Descriptive charts. The first descriptive chart actually adopted by the Society of American Bacteriologists was in 1907. The chart has been revised from time to time and at present there are two forms: one known as the Standard Descriptive Chart and the other as the Descriptive Chart for Instruction. The latter is very much simpler than the former. The former is printed on both sides of an 8½- by 11-in. sheet of light cardboard; the latter on a sheet of heavy paper of the same size.

The object of the descriptive chart is to provide a space for recording the most important characteristics of a single culture. The Standard Chart is the most complete and is intended especially for advanced work in bacteriology. Unfortunately, however, it does not meet modern research needs at all perfectly because each group of bacteria requires its own set of tests and no form can be drawn up sufficiently detailed to cover all of them. The Chart for Instruction, on the other hand, is so much simpler and contains so much blank space that it sometimes is found to be more satisfactory in research work than the Standard Chart. It is, however, intended primarily for students to use in characterizing cultures furnished them in connection with their classwork.

Manual of Microbiological Methods. The origin of the present manual traces back to a committee report which was printed in the *Journal of Bacteriology* and was distributed in reprint form by the committee (1918). It was only 14 pages long and covered only the methods used in carrying out the determinations called for on the descriptive chart of those days. After one or two minor revisions it was converted in 1923 into the "Manual of Methods for Pure Culture Study of Bacteria," which as remarked above was published in loose-leaf form until 1956. The first edition was only 48 pages in length and, like its predecessor, was confined wholly to the methods needed in using the chart. Gradually, however, it was

expanded until it included 10 leaflets, and it has come to include a variety of methods other than those called for in the use of the descriptive chart. By 1953 several other subjects had been selected as desirable to include in future editions, and plans were made for converting the manual into a larger publication.

While the old manual was in loose-leaf form, it was kept up to date by periodic revision of its leaflets, one or two at a time, and by means of a continuation service owners were enabled to secure the latest editions to insert in their copies. This feature now, unfortunately, has to be given up because of the increased size of the book and of certain practical difficulties involved in loose-leaf publication. The committee regrets the necessity of abandoning the old system, but there seems to be no other course than to convert it into a regular book and to hope that revisions to bring the contents up to date can be accomplished by means of periodic new editions.

HISTORICAL

The first efforts toward producing a descriptive chart for characterizing bacteria were made by two different individual investigators, H. W. Conn and S. de M. Gage. The work of these two investigators called the matter to the attention of bacteriologists in general, and it was finally brought before the Society of American Bacteriologists by F. D. Chester at the Philadelphia meeting in December, 1903, and then again at the 1904 meeting, when he explained his idea of a "group number" which would be descriptive of the salient characters of an organism. On his recommendation the society appointed a Committee on Methods for the Identification of Bacterial Species of which Professor Chester was made chairman. This committee drew up the first descriptive chart with which the Society of American Bacteriologists had any connection.

This chart was put before the society at its 1905 meeting. It was presented at this time as a preliminary effort, and no endorsement of it was given by the society, nor apparently was such endorsement requested. The committee was instructed to continue its work, and a second chart was prepared during 1906 and presented at the society meeting in December of that year. At this meeting it was decided that the chart should call for more complete data concerning bacteria than provided for by either of the two charts already submitted, so the committee was instructed to do further work along this same line.

The committee at this time was composed of F. D. Chester, F. P. Gorham, and E. F. Smith, but Professor Chester was largely responsible for the first two charts presented at society meetings. Before the committee undertook a further revision, however, he had left bacteriological work and hence was no longer active on the committee. During 1907, therefore, Dr. Smith acted as chairman of the committee, and under his supervision the committee drew up another chart which was presented to the society at its meeting in December of that year. This chart was officially endorsed by the society and was put on sale by the secretary of the society.

For several years following no changes were made in the chart. The next step in its development was brought about by H. A. Harding (1910), who published a paper in which he outlined the complete history of the chart, with copies of the early charts,

and discussed improvements that might be made. This paper is available for those desiring more detail concerning this early history than is given here.

As the society felt that further modifications were now needed, a new committee was appointed in 1911 consisting of F. P. Gorham, C. E. A. Winslow, Simon Flexner, H. A. Harding, and E. O. Jordan. This committee gave a report at the 1913 meeting, presenting a chart which was put on sale by the society but was not officially endorsed. As this committee was unable to continue the work, an entirely new one was appointed at this time consisting of H. A. Harding, H. J. Conn, Otto Rahn, W. D. Frost, and L. J. Kligler. This committee soon lost Dr. Rahn, who left the country in 1914, and M. J. Prucha was added in his place. The committee was called the Committee on Revision of the Chart for the Identification of Bacterial Species.

The new committee was instructed by the society to make a conservative revision of the chart and at the same time to draw up a manual of methods to be used in connection with it. At the 1914 meeting of the society, therefore, a chart was presented for approval, much like the 1907 chart except for its more logical arrangement of data. This chart was given the society's endorsement and was issued during 1915.

The 1914 chart was printed on a sheet with its back entirely blank, the glossary previously on the back having been omitted. The committee gave as the reason for this that the glossary would be included in the manual on methods shortly to be published. The publication of this manual was delayed, however, pending investigation of the methods to be included in it. This investigation of methods was to be undertaken not only for the sake of the manual but also as a preliminary step toward radical revision of the chart, which was felt to be badly needed. Early in 1917, however, and before this program could be carried out, the chairman of the committee was forced by pressure of other duties to drop the work. As he wished to remain on the committee, however, no change in membership was made, but H. J. Conn was asked to become chairman.

The committee then undertook the first step toward the preparation of a manual on methods. A report was presented at the 1917 meeting, giving the methods recommended at that time for use with the chart. The report was printed in the *Journal of Bacteriology*, March, 1918, and was subsequently sold by the society in the form of reprints. This report was considered a preliminary manual on methods.

The committee proposed at the same time a much simplified chart in the form of a four-page folder, which it recommended for use in instruction until the official chart could be given the revision it needed. This chart was not endorsed by the society but was printed and sold by the society for two or three years.

This same committee (but now called the Committee on the Descriptive Chart) issued another report on methods which appeared in the *Journal of Bacteriology*, March, 1919, dealing with the gram stain, production of acid, and the reduction of nitrates. At the 1919 meeting it issued a further report which appeared in the *Journal of Bacteriology* in two parts, March and May, 1920. The first part of the report was a revision of the one which had been published in March, 1918, and was sold as a revised manual of methods until the reprints were exhausted in 1922.

At the 1920 meeting the Committee on the Descriptive Chart was discharged with the understanding that its functions would be taken over by a committee of broader scope then appointed and called the Committee on Bacteriological Technic. This committee was appointed with the understanding that its membership should fluctuate from year to year in order to keep on it men actively interested in the work.

The new committee made a further revision of the chart, which was presented at the 1920 meeting and endorsed by the society. Later editions of this chart have been drawn up by the committee but have not been submitted to the society for official endorsement. In order to avoid committing the society in favor of any of the methods

concerned, recent editions of the chart have merely been presented by the committee and permission asked to put them on sale.

The committee issued four further reports in the *Journal of Bacteriology* (1921, 1922*a*, *b*, and *c*) before the manual was prepared. One of these reports (1922*b*) proposed certain revisions of methods in the case of the gram stain, fermentation, nitrate reduction, indole and hydrogen sulfide production. The committee presented this report at the 1922 meeting of the society with the recommendation that the revised material be published as part of a "Manual of Methods for Pure Culture Study of Bacteria." The committee was thereupon instructed by the society to publish this manual, using the loose-leaf form of binding, with the understanding that new folders be issued from time to time to keep it up to date. This was done, and the system continued till 1956, when, as explained above, it proved necessary to convert the manual into book form and its name was changed to "Manual of Microbiological Methods."

The Committee on Bacteriological Technic has seen the following changes in personnel:

1920	H. J. Conn,[1] K. N. Atkins, I. J. Kligler, J. F. Norton, G. E. Harmon.
1921	H. J. Conn,[1] K. N. Atkins, G. E. Harmon, Frederick Eberson, Alice Evans.
1922	H. J. Conn,[1] K. N. Atkins, G. E. Harmon, Frederick Eberson, F. W. Tanner, and S. A. Waksman.
1923	H. J. Conn,[1] K. N. Atkins, J. H. Brown, G. E. Harmon, G. J. Hucker, F. W. Tanner, and S. A. Waksman.
1924–5	H. J. Conn,[1] K. N. Atkins, J. H. Brown, Barnett Cohen, G. J. Hucker, F. W. Tanner.
1926–7	H. J. Conn,[1] Barnett Cohen, Elizabeth F. Genung, W. L. Kulp, W. H. Wright; with G. J. Hucker and S. Bayne-Jones as a subcommittee on serological methods.
1928	H. J. Conn,[1] Victor Burke, Barnett Cohen, Elizabeth F. Genung, W. L. Kulp, W. H. Wright.
1929–30	H. J. Conn,[1] Victor Burke, Barnett Cohen, Elizabeth F. Genung, I. C. Hall, W. L. Kulp, W. H. Wright (deceased, May, 1929).
1931–4	H. J. Conn,[1] Barnett Cohen, Elizabeth F. Genung, Victor Burke, I. C. Hall, J. A. Kennedy.
1935	H. J. Conn,[1] Victor Burke, Barnett Cohen, M. W. Jennison, J. A. Kennedy.
1936–42	H. J. Conn,[1] J. H. Brown, Victor Burke, Barnett Cohen, C. H. Werkman, M. W. Jennison, J. A. Kennedy, A. J. Riker.
1943–5	H. J. Conn,[1] Victor Burke, Barnett Cohen, C. H. Werkman, M. W. Jennison, J. A. Kennedy, L. S. McClung, A. J. Riker.
1946–7	H. J. Conn,[1] G. H. Chapman, Barnett Cohen, I. C. Gunsalus, M. W. Jennison, L. S. McClung, A. J. Riker, C. E. ZoBell.
1948	M. W. Jennison,[1] G. H. Chapman, Barnett Cohen, H. J. Conn, I. C. Gunsalus, J. A. Kennedy, L. S. McClung, A. J. Riker, C. A. Stuart, C. E. ZoBell.
1949	M. W. Jennison,[1] G. H. Chapman, H. J. Conn, I. C. Gunsalus, L. S. McClung, C. A. Stuart, A. J. Riker, C. E. ZoBell.
1950	M. W. Jennison,[1] R. C. Bard, G. H. Chapman, H. J. Conn, I. C. Gunsalus, L. S. McClung, C. A. Stuart, A. J. Riker, C. E. ZoBell.
1951–2	M. W. Jennison,[1] R. C. Bard, G. W. Burnett, H. J. Conn, H. C. Lichstein, L. S. McClung, A. P. McKee, M. J. Pelczar, A. J. Riker, C. A. Stuart, C. E. ZoBell.

[1] Chairman.

1953–4 M. J. Pelczar,[1] R. C. Bard, G. W. Burnett, H. J. Conn, E. E. Evans M. W. Jennison, H. C. Lichstein, L. S. McClung, A. P. McKee, A. J. Riker, J. Warren, O. B. Weeks, F. A. Weiss.

1955–7 M. J. Pelczar,[1] R. C. Bard, G. W. Burnett, H. J. Conn, R. D. DeMoss, E. E. Evans, M. W. Jennison, A. P. McKee, A. J. Riker, J. Warren, O. B. Weeks, F. A. Weiss.

GENERAL CONSIDERATIONS

Pitfalls to Be Avoided by the Student

In studying microbial cultures with the object of identifying them or describing them, the student is apt to run into certain pitfalls. Some of these apply specifically to certain types of work and are therefore best taken up in the various chapters of this book where they seem properly to fit. Others are more general; some, in fact, are well known even to beginning students in bacteriology. However, as others are less fully appreciated, a few words concerning some of these pitfalls seem called for here—even at the risk of repeating cautions that may seem too elementary. These pitfalls arise primarily from three sources: (1) the danger of impure cultures, (2) confusing results because of variation of bacterial species, (3) differences in methods of study.

The danger in impure cultures is, of course, thoroughly understood. Unfortunately, however, the second consideration just mentioned makes it more important to emphasize the danger of impure cultures today than was the case before 1920. In those days bacteriologists quite generally accepted the idea of monomorphism, and whenever a culture was observed to be noticeably abnormal in either morphology or physiology, it was promptly discarded as a contaminant. When, however, it began to be learned that even the most strictly guarded pure cultures might show changes in morphology during their life history, and then later when it was realized that the same organism might occur in two or more phases showing distinctly different cultural and physiological characteristics, the old ideas of monomorphism were decidedly upset. As a result of the changing point of view, it is very easy for a careless student today to believe that he is observing two phases of the same pure culture when, actually, one of his "phases" is a contaminant. This makes constant checking as to purity of cultures even more important than it was before dissociation into phase variants was generally accepted by bacteriologists.

Accepting the idea of dissociation presents other difficulties to the student. Without exhaustive study, it is sometimes very easy to describe two phases of the same species as though they were different organisms. It is also easy to prepare a description of some culture which is an illogical jumble of the characteristics of two or more phases, due to

[1] Chairman.

the fact that it was first studied in an unstable form and dissociation was taking place during the course of the study. On the other hand, some of the methods employed in the hopes of inducing phase variation may actually cause contamination and be incorrectly interpreted. Some of these points are very adequately discussed by Frobisher (1933).

The third source of error mentioned above (variation in methods) also needs emphasis. When a species is described in such terms as one frequently encounters in published descriptions, e.g., "produces acid (without gas) from glucose and lactose but not from sucrose; does not reduce nitrates," one has to guess at the answers to such questions as these: What basal medium was used in each instance? What indicator of acid production was employed? How thorough a study was made to show the absence of any acid from sucrose or of any reduction of nitrate? Or, in the last instance, is it safe to assume that the author of the species merely failed to find nitrite in some nitrate medium? Unless such questions are answered correctly, the description is meaningless; the attempt to identify an unknown culture with such a description may well give misleading results.

With all these pitfalls to avoid, it is easy to see how the same set of data, no matter how carefully prepared, can be differently interpreted by two different bacteriologists. As a result extreme caution is urged, both in determining the identity of a culture and in deciding whether or not to pronounce it a new species.

Practical Hints

Determining the characteristic of a culture. One should always, if possible, make a complete study of a culture promptly after its first isolation while it is in a condition to display its true characteristics. When a culture has been carried in the laboratory for a long period of time, it may change in some respects from the original. When practical, such cultures should be exposed to conditions which might bring them back to the "normal." When this is done, however, the possibility should always be recognized that by such manipulation dissociation may be induced so that the phase subsequently studied may be quite different from the original isolation. Whenever distinct evidence of dissociation is observed, each phase should be studied and recorded separately, and efforts should be made to reverse the change or to obtain the same change with other strains until the possibility of impure cultures seems to be out of the question. No importance should ever be attached to a single determination unless supported by replications giving the same results. In describing morphology, one should not be contented with one or two observations but should study several transfers and should follow up each of them day by day for about a week. When changes are observed, a careful

study should be made to learn whether they indicate morphologic variation, dissociation, or merely contamination. In making special staining tests, like the gram stain, several determinations should be made on separate transfers of the culture and at different ages, because there are species that vary in their staining reactions and such variation cannot be detected by single determinations. As a check on the technic, a known positive and a known negative culture should be included in the study. For example, when making a gram stain, it is good practice to place on the slide, beside the culture under study, a smear containing a mixture of a known gram-positive and a known gram-negative organism (which differ markedly in morphology). Then it is possible to observe if the expected results are obtained with the known cultures and thus to have some degree of control on the technic.

Identification. After recording the characteristics of an organism, the next step is identification, if possible, with a previously described species. This should never be attempted until at least six representative strains of the unknown organism isolated from more than one source, if possible, have been studied. No rules can be given for identifying the culture. Descriptions of bacteria are scattered so widely through the literature and vary so greatly in their form that identification is often extremely different. Bergey's "Manual of Determinative Bacteriology" is a great help, but it is usually necessary to go back to original descriptions and often to secure transfers of authentic strains before certain identification can be made. Difficult as this procedure is, no one is justified in naming a new species of bacteria until a comprehensive search through the literature of species already described has been made. Frequently it is necessary to refer in some publication to a previously described species on the basis of such an identification as this. In this case it is important to state in the publication whether or not an authentic strain of the species has been obtained for comparison; if so, from where obtained; if not, what published description of the species was followed in making the identification. As to a name to use for such a species, one may follow the original author's nomenclature or may give it the name employed in some modern system (e.g., Bergey). Whatever name is chosen, no confusion will result if it is accompanied by the name of the original author of the specific name and by that of the one making the combination of generic and specific names. Thus, whether one says "*Bacillus coli* Migula" or "*Escherichia coli* (Migula) Castellani and Chalmers," it is entirely clear what species is intended.

Naming a new species. When it proves impossible to identify a culture with any species described in the literature, it is often desirable to publish a description of it as a new species. When publishing such a description, there are five important points to be kept in mind:

1. The description should be based on at least six representative isolations of the organism.
2. If variations are found to occur among these strains, a critical study must be made to be sure that they are not the result of contamination.
3. In naming any characteristic of the species, especially if it is a negative character (e.g., "nitrates not reduced"), the technic by which it is determined must be stated.
4. Before giving the results of any test as positive or negative, comparisons must be made with a control culture known to be positive and one known to be negative.
5. Before actually assigning a name one should consult a specialist in bacterial taxonomy, both as to the necessity for a new name and as to the validity of the name selected. The Board of Editor-Trustees of Bergey's Manual, for example, are always very glad to offer such advice.

If these hints were followed by all who are trying to identify species or to publish descriptions of them, much of the confusion in bacterial nomenclature would be eliminated.

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CHAPTER II

Staining Methods

H. J. Conn in collaboration with J. W. Bartholomew and M. W. Jennison

GENERAL PRINCIPLES

The staining of bacteria depends in general upon the same properties of dyes as does the staining of animal or plant tissue for histological purposes. Short discussions of the nature of dyes, with special reference to staining are given elsewhere (Conn, 1953), and only the briefest summary of the subject need be given here.

All bacterial dyes are synthetic products—anilin dyes, or coal-tar dyes, as they are generally called. Although the synthetic dyes vary greatly in their chemical nature and staining properties, they are for practical purposes often divided into two general groups, the acid dyes and the basic dyes. These terms do not mean that the dyes in question are free acids or free bases. The free color acids and bases, when obtainable, are colored, to be sure, but they are often insoluble in water and rarely have appreciable staining action; i.e., the colors do not "stick." The salts of these compounds, on the other hand, are more soluble, penetrate better, and stain more permanently; they are the true dyes.

An acid dye is the salt of a color acid; a basic dye the salt of a color base. In other words, acid dyes owe their colored properties to the anion, basic dyes to the cation. The actual reaction of an aqueous solution of a dye, however, depends on several factors, and an acid dye may well be basic in reaction, while a basic dye may be acid. This is because the reaction of such a solution depends on the relative strengths of the dye ion and of the anion or cation with which it is combined in the dye salt.

Basic dyes have greatest affinity for the nuclei of cells, probably because of the acid nature of the nuclear material. Acid dyes have a stronger tendency to combine with the cytoplasm. Bacteria do not show typical cell structure, and they tend to stain fairly uniformly with nuclear, i.e.,