Identification of Enterobacteriaceae

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Third Edition

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The third edition of this book is dedicated to all those who found the earlier editions of value to them in their work.

Preface

This new edition was discussed with the late Dr. P. R. Edwards during 1965 and prior to his death on May 16, 1966. The decisions reached in those discussions regarding the general format, contents, nomenclature, and other important points have been followed in the preparation of this edition.

The intent is not to prescribe rules as to the media and methods that must be used by any worker. However, there are certain minimum standards below which work with Enterobacteriaceae should not fall, and there are a number of methods and media which have proved satisfactory in the hands of numerous competent and experienced workers. Some methods and media have become outmoded, while certain newer media and methods require evaluation. The purpose of this text is to provide recommended methods that may be employed in the isolation and study of Enterobacteriaceae, and to point out some pitfalls which the worker in even the smallest laboratory may avoid.

Although systematic bacteriology is neglected by most and complained about by many, it is essential for identification and communication. Labels of some sort must be available for the bacteria, and there must be a considerable amount of agreement regarding these labels and their use. Therefore, there must be rules of procedure for the selection and usage of such labels. Such rules should, however, be sufficiently flexible to allow for reasonable, substantiated, and justified change, for systematic bacteriology is an evolving discipline which should be permitted to continue its evolution.

The system of nomenclature and taxonomy employed in this book is a result of that evolution and is based on examination of the biochemical reactions of large numbers of cultures. It is felt that biochemical reactivities must form the primary basis for classification at the level of the tribe and the genus within the family Enterobacteriaceae. If we consider the community of antigens within the family and the intensive intergeneric antigenic relationships that are known, the need for the use of biochemical rather than serological reactions for classification at these levels is clear, and, with a few possible exceptions, this also holds true for classification at the species level.

The systematics of Enterobacteriaceae should continue to evolve, and it is reasonable to suppose that future generations of bacteriologists will continue to improve upon systems currently in use. Therefore, the best that present-day investigators can hope for is to provide a sound basis so that changes necessary in the future can be minimal and can be made gradually.

The discussions of the isolation of salmonellae from foods and feeds and of the bacteriophage typing of *Salmonella typhi* which appeared in the second edition have been omitted.

This edition contains a large amount of data on the biochemical reactions given by members of the genera of Enterobacteriaceae. These data are summarized in tabular form and the percentages of positive reactions, etc., are given for each substrate or test. Tests of particular value for differentiation of members of related genera and species are also tabulated. The chapters and their contents are arranged in such a way that the personnel of all laboratories of medical and public health bacteriology should be able to select tests and methods for presumptive identification, through intermediate stages, to complete biochemical characterization of an isolant. Partial or complete serological analyses may also be made in many instances.

Investigation of the antigenic properties of members of several of the genera within the family has been extended. Additional antigens and numerous serotypes have been characterized in genera previously studied intensively. In other genera where little or no work on antigenic properties had been done, knowledge has been expanded. While the antigenic schemata that have been established are not given in detail in every instance, references to advances are included and the work is described briefly. Until such time as it is demonstrated that serologic typing within a given genus or species can be used profitably in the study of infections, it does not seem worthwhile to catalogue long lists of antigens, type strains, and serotypes for all known schemata.

Discussion of several subjects is included in a new chapter (Chapter 4). Since the bases for serologic typing within the several genera of the family Enterobacteriaceae are similar, and since several of the varia-

tional phenomena that affect serotyping within the various genera are very much the same, discussion of these has been placed in the new chapter. Brief reviews of the literature on the immunochemistry of the antigens of Enterobacteriaceae and the literature that deals with the genetic changes that may affect the results of serotyping are included. Fluorescent antibody techniques have been applied to a variety of Enterobacteriaceae, and studies on the resistance (R) factors have been extended to many kinds of Enterobacteriaceae, as well as to other bacteria, hence these are mentioned in Chapter 4. Incorporation of these subjects in a single chapter avoids repetition in other chapters and provides additional basic information with which some readers may not be particularly familiar. In some instances this information is essential to the understanding and interpretation of results of serological analyses. The seemingly unrelated subject of the R factors is included because it is felt that all who work with Enterobacteriaceae should be at least casually acquainted with the subject.

The references cited should be sufficient to enable the reader to begin an independent search of the literature. Every care has been taken to eliminate errors, but both the author and the publisher shall be glad to be notified of any errors found by readers.

The importance of accurate identification of members of all genera of Enterobacteriaceae cannot be overstated. It is hoped that this text will prove to be a useful tool in this work.

Atlanta, Georgia
March, 1972

W. H. Ewing

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Chapter 1
Taxonomy and Nomenclature

INTRODUCTION

The classification and nomenclatural system for the family ENTEROBACTERIACEAE used in this edition (v. inf.) is that proposed by Ewing (1963), as emended (1966, 1967, 1969, and 1970). This system resulted from comparative studies of the biochemical reactions given by relatively large numbers of cultures of each of the genera (e.g., Ewing, 1968).

From the beginning, the aim of the above-mentioned investigations was to compile numerical and percentage data that could be used to compare *all* of the reactions given by members of one genus with those of another, as well as to provide means for differentiation of genera and species. Therefore, the genera *Escherichia* and *Shigella* are placed in the same tribe because biochemically these two genera are more closely related to each other than either is related to any other genus in the family. The genera *Salmonella, Arizona,* and *Citrobacter* are placed in the same tribe for the same reason; and so on, throughout the family. It is the author's opinion that classification of the bacteria in this manner, i.e., on the basis of overall similarities, is much more logical and reasonable than classifications based on a single character, or on one or two criteria. As examples, *Shigella* and *Salmonella* are *not* placed in the same tribe because they share a negative character (failure to ferment lactose rapidly), as they are in the 7th edition of *Bergey's Manual* (Breed, Murray, and Smith, 1957). Further, a tribe SERRATEAE is not recognized, since pigment production on ordinary media is not a cardinal characteristic of *Serratia* (Davis et al., 1957 et seq.). The tribe ERWINEAE is not recognized as such, although part of it (the genus *Pectobacterium*) is incorporated into the tribe KLEBSIELLEAE for reasons given by Graham (1964) and Ewing (1967). The genus *Alginobacter* is not recognized because ENTEROBACTERIACEAE do not liquefy alginate and for other reasons (Davis and Ewing, 1964). At this late date it should not be necessary to add anything about the "genus" *Paracolobactrum* or the "paracolon group," since these have been dealt with by many investigators (e.g., Fields et al., 1967). However, for the record, the author will repeat the statement made in the second edition of this book: "The establishment of a single genus *[Paracolobactrum]* composed of diverse forms simply upon the basis of delayed fermentation of lactose is completely unjustified. For example, a culture classified as *Paracolobactrum coliforme* may be changed into a typical *Escherichia coli* culture simply by selection of components that rapidly ferment lactose."

The several classifications of ENTEROBACTERIACEAE proposed by Kauffmann (e.g., 1956, 1963, 1966) differ taxonomically, nomenclaturally, and in concept from that employed herein. However, it is not the author's intent to debate the merits of one system over another. The system employed in this edition is used not so much because it was proposed by the author as because it appears to be the best available at this time. Further, as has been said many times (Ewing, 1963, and since), acceptance of any system comes from usage (no system can be legislated), and the classification used herein is gaining wide acceptance in this hemisphere, at least. To the author's knowledge the nomenclature employed is correct. With one exception, the emendations mentioned above, and made since 1963, involve changes in certain citations, and a specific epithet, necessitated by actions of the Judicial Commission of the International Committee on Nomenclature of Bacteria. The exception is the addition of the tribe EDWARDSIELLEAE (Ewing et al., 1965).

An outline of the nomenclature of the family ENTEROBACTERIACEAE, and definitions (Ewing, 1967; emended 1970) for the family, its tribes, and genera follow.

The Nomenclature of the Family ENTEROBACTERIACEAE

Family ENTEROBACTERIACEAE Rahn

Tribe I ESCHERICHIEAE Bergey, Breed, and Murray

Genus I *Escherichia* Castellani and Chalmers

1. *Escherichia coli* (Migula) Castellani and Chalmers

Genus II *Shigella* Castellani and Chalmers
1. *Shigella dysenteriae* (Shiga) Castellani and Chalmers
2. *Shigella flexneri* Castellani and Chalmers
3. *Shigella boydii* Ewing
4. *Shigella sonnei* (Levin) Weldin

Tribe II EDWARDSIELLEAE Ewing and McWhorter

I *Edwardsiella* Ewing and McWhorter
1. *Edwardsiella tarda* Ewing and McWhorter

Tribe III SALMONELLEAE Bergey, Breed, and Murray

Genus I *Salmonella* Lignières
1. *Salmonella cholerae-suis* (Smith) Weldin
2. *Salmonella typhi* (Schroeter) Warren and Scott
3. *Salmonella enteritidis* (Gaertner) Castellani and Chalmers

Genus II *Arizona* Ewing and Fife
1. *Arizona hinshawii* (Ewing and Fife) Ewing

Genus III *Citrobacter* Werkman and Gillen
1. *Citrobacter freundii* (Braak) Werkman and Gillen

Tribe IV KLEBSIELLEAE Trevisan

Genus I *Klebsiella* Trevisan
1. *Klebsiella pneumoniae* (Schroeter) Trevisan
2. *Klebsiella ozaenae* (Abel) Bergey, Breed, and Murray
3. *Klebsiella rhinoschleromatis* Trevisan

Genus II *Enterobacter* Hormaeche and Edwards
1. *Enterobacter cloacae* (Jordan) Hormaeche and Edwards
2. *Enterobacter aerogenes* (Kruse) Hormaeche and Edwards
3. *Enterobacter hafniae* (Moeller) Ewing
4. *Enterobacter liquefaciens* (Grimes and Hennerty) Ewing

Genus III *Pectobacterium* Waldee
1. *Pectobacterium carotovorum* (Jones) Waldee

Genus IV *Serratia* Bizio
1. *Serratia marcescens* Bizio *(Serratia marcescens* subspecies *marcescens)*
1a. *Serratia marcescens* (subspecies *kiliensis* Lehmann and Neumann) Ewing, Davis, and Johnson

Tribe V PROTEEAE Castellani and Chalmers

Genus I *Proteus* Hauser
1. *Proteus vulgaris* Hauser
2. *Proteus mirabilis* Hauser
3. *Proteus morganii* (Winslow, Kligler, and Rothberg) Rauss
4. *Proteus rettgeri* (Hadley et al.) Rustigian and Stuart

Genus II *Providencia* Ewing
1. *Providencia alcalifaciens* (DeSalles Gomes) Ewing
2. *Providencia stuartii* (Buttiaux et al.) Ewing

Note: The first species listed in each genus is the type species.

The system given in the outline has been used by the author and colleagues for many years. Further, it has been adopted by the editors of certain manuals, by the author of at least one textbook, and it has appeared in a number of articles in well-known American journals. Therefore it may be recommended that laboratorians use it. Further, it is recommended that they inform physicians, nurses, epidemiologists, and others with whom they come in contact concerning the system so these persons can become familiar with it. Since there is very little that is new in the system, such familiarization should not take long or be difficult.

Only three species of *Salmonella* are recognized. The concept of limitation of the number of species of *Salmonella* to three apparently originated with Borman et al. (1944). This concept also was adopted by Kauffmann and Edwards (1952). Although alternatives were discussed, the three species concept was used by Ewing (1963). The late Dr. P. R. Edwards agreed, and stated (1962) that the proposal made with Professor Kauffmann in 1952 still was his choice as the most logical solution to this problem (see 1963 publication cited above). There is ample precedent for limiting the number of species in a genus. For example, only four species are recognized in the genus *Shigella* (see Chapter 6).

The three species recognized in the genus *Salmonella* are *Salmonella cholerae-suis* (the type species), *Salmonella typhi,* and *Salmonella enteritidis.* The name *S. enteritidis* was chosen for the third species because the epithet *enteritidis* was the oldest validly published (1888), legitimate epithet other than *cholerae-suis* and *typhi.* All salmonellae other than *S. cholerae-suis* and *S. typhi* are serotypes of *S. enteritidis.* It is emphasized that attention should be given to correct usage. Except where employed in a general way, as in an outline or summary of nomenclature or in a definition of the third species, the name *S. enteritidis* should not be used alone, since 1400 or more serotypes and bioserotypes are included in it. The designations given to the numerous serotypes of *S. enteritidis* are infrasubspecific and they have no standing in nomenclature

(see International Code, 1966). It is of no consequence whether these infrasubspecific terms are italicized, since the use of italics, or other printing device, does not alter their status – they still are infrasubspecific designations, equivalent to, and used in lieu of, antigenic formulas.

Therefore the serotypes (ser) and bioserotypes (bioser) of *S. enteritidis* are recorded in the manner indicated in the examples that follow. The infrasubspecific designations are capitalized for reasons of clarity only. The names of the other two species *(S. cholerae-suis* and *S. typhi)* are unaffected and cultures of these are reported by these names as in the past.

Nomenclature used in this book	Old designation
S. enteritidis ser Typhimurium	*S. typhimurium*
S. enteritidis ser Heidelberg	*S. heidelberg*
S. enteritidis ser Enteritidis	*S. enteritidis*
S. enteritidis bioser Paratyphi-A	*S. paratyphi A*
S. enteritidis bioser Pullorum	*S. pullorum*

The term bioserotype is used in connection with certain serotypes of *S. enteritidis* that possess unique biochemical characteristics (see Chapter 8). Unnamed serotypes are recorded as in the following example: *S. enteritidis* ser 58:a:-.

It is recommended that in formal publications, authors give the complete name of each species (e.g., *Salmonella enteritidis*) the first time that each occurs. Thereafter, reference to serotypes of *S. enteritidis* may be made as follows: ser Enteritidis, ser Typhimurium, bioser Paratyphi-A, etc. Certain strains of serotypes of *Salmonella* used in research and for other purposes may be distinguished by numerals or letters following the serotypic designation (e.g., *S. enteritidis* ser Senftenberg 775W).

At first glance the above-mentioned system of nomenclature for the genus *Salmonella* may appear cumbersome. However, if it is used in the manner suggested it is simple and easy to use and has many advantages, as illustrated in Chapters 8 and 9. The author has adopted the three-species concept because it is practical, because no better system has been proposed, and because it is legitimate according to the rules of nomenclature. Further, the author is of the opinion that it is better to adopt and use a legitimate system than to continue indefinitely the use of an illegitimate one that appears to accord species status to all serotypes of salmonellae. Some workers may have initial difficulty in differentiating between the species *S. enteritidis* and the serotype Enteritidis (= 9, 12:g,m:-). The author believes that this initial difficulty will be overcome in a short time as workers become familiar with the system. However, it probably would be better to conserve the epithet *enterica* against the epithet *enteritidis* in the name of the third species of *Salmonella,* as suggested by Ewing (1963). If this were done, the name of the third species would be *Salmonella enterica* and possible confusion of the sort mentioned would be obviated. Such conservation requires action by the Judicial Commission of the International Committee on Nomenclature of Bacteria.

Although the author has adopted the three-species concept of salmonellae for use herein, it should go without saying that any individual may use any system that is to his liking. The use of the above-mentioned system in this book should not detract in any way from whatever other value the book may have.

DEFINITIONS FOR THE FAMILY ENTEROBACTERIACEAE, ITS TRIBES, AND GENERA

The Family ENTEROBACTERIACEAE Rahn

The family ENTEROBACTERIACEAE consists of gram-negative, aerobic, facultatively anaerobic, asporogenous, rod-shaped bacteria that grow well on artificial media. Some species are atrichous, and nonmotile variants of motile species also may occur. Motile forms are peritrichously flagellated. Nitrates are reduced to nitrites, and glucose is utilized fermentatively with the formation of acid or of acid and gas. The indophenol oxidase test is negative and alginate is not liquefied. Pectate is liquefied by members of only one genus *(Pectobacterium).*

Tribe I ESCHERICHIEAE Bergey, Breed, and Murray

ESCHERICHIEAE are motile or nomotile bacteria that conform to the definition of the family ENTEROBACTERIACEAE. The methyl red reaction is positive and the Voges-Proskauer test is negative. Urease, phenylalanine deaminase, and hydrogen sulfide are not produced; sodium malonate is not utilized, gelatin is not liquefied, and growth does not occur on Simmons' citrate agar nor in medium containing potassium cyanide.

Genus I *Escherichia* Castellani and Chalmers

The genus *Escherichia* is composed of motile or nonmotile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe ESCHERICHIEAE. Both acid and gas are formed from a wide variety of fermentable carbohydrates but anaerogenic biotypes occur. Salicin is fermented by the majority of cultures but adonitol and inositol are utilized infrequently. Lactose usually is fermented rapidly but some strains utilize it slowly and some fail to ferment this substrate. Lysine, arginine, and ornithine are decarboxylated by the majority of cultures, acid is formed from sodium mucate, and sodium acetate is utilized as a sole source of carbon. The type species is *Escherichia coli* (Migula) Castellani and Chalmers.

Genus II *Shigella* Castellani and Chalmers

The genus *Shigella* is composed of nonmotile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe ESCHERICHIEAE. With the exception of certain biotypes of *Shigella flexneri* 6, visible gas is not formed from fermentable carbohydrates. Compared with *Escherichia, Shigella* are less active in their utilization of carbohydrates. Salicin, adonitol, and inositol are not fermented. Strains of *Shigella sonnei* ferment lactose upon extended incubation, but other species do not utilize this substrate in conventional medium. Lysine is not decarboxylated, the majority of strains do not possess a demonstrable arginine dihydrolase system, and ornithine is decarboxylated only by *S. sonnei* and *Shigella boydii* 13. The type species is *Shigella dysenteriae* (Shiga) Castellani and Chalmers.

Tribe II EDWARDSIELLEAE Ewing and McWhorter

EDWARDSIELLEAE are motile bacteria that conform to the definition of the family ENTEROBACTERIACEAE. Hydrogen sulfide is produced abundantly, indol is formed, the methyl red test is positive, the Voges-Proskauer reaction is negative, phenylalanine is not deaminated, and urea is not hydrolyzed. Gelatin is not liquefied, growth does not occur in Simmons' citrate medium, nor sodium acetate medium, nor in medium containing potassium cyanide. Lipase is not formed and arginine dihydrolase is not produced. Esculin is not hydrolyzed, and erithritol, and adonitol are not fermented.

Genus *Edwardsiella* Ewing and McWhorter

The genus *Edwardsiella* is composed of motile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe EDWARDSIELLEAE. Lysine and ornithine are decarboxylated, but neither malonate nor mucate is utilized. Glucose and maltose are fermented promptly, and with rare exceptions gas is formed from these substrates. Glycerol is utilized slowly by the majority of strains, but lactose, sucrose, mannitol, dulcitol, salicin, inositol, sorbitol, raffinose, rhamnose, xylose, cellobiose, and alpha methyl glucoside are not attacked. The type species is *Edwardsiella tarda* Ewing and McWhorter.

Tribe III SALMONELLEAE Bergey, Breed, and Murray

SALMONELLEAE are motile bacteria that conform to the definition of the family ENTEROBACTERIACEAE. The methyl red reaction is positive, the Voges-Proskauer test is negative, indol is not formed, and phenylalanine is not deaminated. Gelatin is not liquefied rapidly in nutrient medium. With few exceptions hydrogen sulfide is produced abundantly, growth occurs on Simmons' citrate and sodium acetate media, arginine dihydrolase is formed, and gas is formed from fermentable carbohydrates. Esculin is not hydrolyzed and erythritol and adonitol are not fermented.

Genus I *Salmonella* Lignières

The genus *Salmonella* is composed of motile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe SALMONELLEAE. Urease is not produced, sodium malonate is not utilized, gelatin is not liquefied, and growth does not occur in medium containing potassium cyanide. Lysine, arginine, and ornithine are decarboxylated. Acid is produced in Jordan's tartrate medium. Dulcitol is fermented and inositol is utilized by numerous strains. Sucrose, salicin, raffinose, and lactose are not fermented. The type species is *Salmonella cholerae-suis* (Smith) Weldin.

Genus II *Arizona* Ewing and Fife

The genus *Arizona* is composed of motile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe SALMONELLEAE. Urease is not produced and growth does not occur in medium containing potassium cyanide. Lysine, arginine, and ornithine are decarboxylated, sodium malonate is utilized, gelatin is liquefied slowly in nutrient medium, and lactose is fermented by the majority of cultures. With few exceptions acid is not produced in Jordan's tartrate medium. Dulcitol and inositol are not fermented and salicin is utilized infrequently. The type species is *Arizona hinshawii* (Ewing and Fife) Ewing.

Genus III *Citrobacter* Werkman and Gillen

The genus *Citrobacter* is composed of motile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe SALMONELLEAE. Lysine is not decarboxylated and less than 20 percent of strains possess ornithine decarboxylase. Urease is produced slowly by the majority of cultures, but the reactions are weak. Growth occurs in medium that contains potassium cyanide and acid is produced in Jordan's tartrate medium. Gelatin is not liquefied in nutrient medium. Dulcitol and cellobiose are fermented rapidly by the majority of cultures. Lactose is utilized but the reactions frequently are delayed. The type species is *Citrobacter freundii* (Braak) Werkman and Gillen.

Tribe IV KLEBSIELLEAE Trevisan

KLEBSIELLEAE are motile or nonmotile bacteria that conform to the definition of the family ENTEROBACTERIACEAE. Hydrogen sulfide is not produced and urea is not hydrolyzed rapidly but delayed reactions may occur. With few exceptions indol is not produced, the methyl red test is negative, and the Voges-Proskauer reaction is positive. Growth occurs in Simmons' citrate medium and in medium containing potassium cyanide. Phenylalanine is not deaminated. Sodium alginate is utilized as a sole source of carbon by certain members of only one genus *(Klebsiella),* and lipase is produced by only one species of *Enterobacter (E. liquefaciens)* and by members of the genus *Serratia.*

Genus I *Klebsiella* Trevisan

The genus *Klebsiella* is composed of nonmotile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe KLEBSIELLEAE. The Voges-Proskauer test is positive, gelatin is not liquefied. Lysine decarboxylase is produced, but arginine dihydrolase and ornithine decarboxylase are not. The majority of cultures utilize sodium alginate as a sole source of carbon and esculin is hydrolyzed. Gas is formed from inositol and glycerol, and by the majority of strains from adonitol. Acid is produced from sorbitol, rhamnose, arabinose, and raffinose. The type species is *Klebsiella pneumoniae* (Schroeter) Trevisan.

Genus II *Enterobacter* Hormaeche and Edwards

The genus *Enterobacter* is composed of motile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe KLEBSIELLEAE. The Voges-Proskauer reaction is positive, gelatin is liquefied slowly by the most commonly occurring forms *(Enterobacter cloacae).* Lysine decarboxylase is not produced by *E. cloacae,* but other species of the genus possess this enzyme system. Ornithine decarboxylase is produced. Sodium alginate is not utilized as a sole source of carbon. Gas is not formed from inositol and glycerol by cultures of *E. cloacae.* Acid is produced from sorbitol, rhamnose, arabinose, and raffinose by the majority of species. One species *(Enterobacter hafniae)* does not ferment sorbitol or raffinose. Only one species *(Enterobacter liquefaciens)* is lipolytic. The type species is *Enterobacter cloacae* (Jordan) Hormaeche and Edwards.

Genus III *Pectobacterium* Waldee

The genus *Pectobacterium* is composed of motile or nonmotile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe KLEBSIELLEAE. Sodium pectate medium is liquefied. A minority of cultures produce indol, but the majority yield positive reactions in the methyl red test. Gelatin is liquefied although the reactions of a minority of strains may be somewhat delayed. Lysine, arginine, and ornithine are not decarboxylated. Sodium alginate is not utilized as a sole source of carbon. Gas is not formed from inositol or glycerol, and adonitol is not fermented. Sorbitol is fermented only very rarely but the majority of cultures produce acid from rhamnose, arabinose, and raffinose. The optimum growth temperature is about 25 C and cultures fail to grow or grow poorly at 37 C. The type species is *Pectobacterium carotovorum* (Jones) Waldee.

Genus IV Serratia Bizio

The genus *Serratia* is composed of motile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe KLEBSIELLEAE. A positive Voges-Proskauer reaction is given by *Serratia marcescens* subsp. *marcescens,* but *S. marcescens* subsp. *kiliensis* gives negative results in this test. Lipase is produced, gelatin is liquefied rapidly, and lysine and ornithine are decarboxylated. Sodium alginate is not utilized as a sole source of carbon. When gas is formed from fermentable substrates the volumes are small (10 per cent or less). Acid is produced from sorbitol but rhamnose, arabinose, and raffinose are not fermented. The type species is *Serratia marcescens* Bizio.

Tribe V PROTEEAE Castellani and Chalmers

PROTEEAE are motile bacteria that conform to the definition of the family ENTEROBACTERIACEAE. The methyl red test is positive and with the exception of occasional strains of *Proteus mirabilis* the Voges-Proskauer reaction is negative. Phenylpyruvic acid is produced rapidly and abundantly from phenylalanine. Growth occurs in medium containing potassium cyanide, but sodium alginate is not utilized as a sole source of carbon. Gas volumes produced from fermentable substrates by aerogenic cultures are small (a bubble to about 15%). Urea is hydrolyzed rapidly and abundantly by members of one genus *(Proteus).*

Genus I *Proteus* Hauser

The genus *Proteus* is composed of motile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe PROTEEAE. Urea is hydrolyzed rapidly. Two species, *Proteus vulgaris* and *Proteus mirabilis,* produce hydrogen sulfide rapidly and abundantly, liquefy gelatin, and swarm on moist agar media. The majority of cultures of these

two species are lipolytic. The other species, *Proteus morganii* and *Proteus rettgeri*, do not possess these particular characteristics. Ornithine is decarboxylated by two species *P. mirabilis* and *P. morganii*. Mannitol is fermented by the majority of strains of *P. rettgeri* but the remaining species fail to produce acid from this substrate. The type species is *Proteus vulgaris* Hauser.

Genus II *Providencia* Ewing

The genus *Providencia* is composed of motile bacteria that conform to the definitions of the family ENTEROBACTERIACEAE and the tribe PROTEEAE. Urea is not hydrolyzed and hydrogen sulfide is not produced. Indol is produced and growth occurs on Simmons' citrate medium. Gelatin is not liquefied, lysine, arginine, and ornithine are not decarboxylated, and lipase is not produced. With rare exceptions mannitol is not fermented and acid is not produced from alpha methylglucoside, erithritol, or esculin. The type species is *Providencia alcalifaciens* (De Salles Gomes) Ewing.

Additional references for citations, earlier reports, etc., may be found in the individual chapters, or in the publications cited.

REFERENCES

Bergey's Manual of determinative bacteriology, 7th ed., 1957. Edited by R. S. Breed, E. G. D. Murray, and N. F. Smith. Baltimore: Williams & Wilkins.

Borman, E. K., C. A. Stuart, and K. M. Wheeler. 1944. J. Bacteriol., **48**, 351.

Davis, B. R., W. H. Ewing, and R. W. Reavis. 1957. Int. Bull. Bacteriol. Nomen. Tax., **7**, 151.

Davis, B. R., and W. H. Ewing. 1964. J. Bacteriol., **88**, 16.

Ewing, W. H. 1963. Int. Bull. Bacteriol. Nomen. Tax., **13**, 95.

_____. 1966. Enterobacteriaceae: taxonomy and nomenclature. CDC Publ.*

_____. 1967. Revised definitions for the family Enterobacteriaceae, its tribes, and genera. CDC Publ.*

_____. 1968. Differentiation of Enterobacteriaceae by biochemical reactions. CDC Publ.* (Revised and emended, 1970.)

Ewing, W. H., A. C. McWhorter, M. R. Escobar, and A. H. Lubin. 1965. Int. Bull. Bact. Nomen. Tax., **15**, 33.

Fields, B. N., M. M. Uwaydah, L. J. Kunz, and M. N. Swartz. 1967. Amer. J. Med., **42**, 89.

Graham, D. C. 1964. Ann. Rev. Phytopathol., **2**, 13.

International Code of Nomenclature of Bacteria. 1966. Int. J. System. Bacteriol., **16**, 459.

Kauffmann, F. 1956. Zentralbl. f. Bakt. I. Orig., **165**, 344.

_____. 1963. Int. Bull. Bacteriol. Nomen. Tax., **13**, 187.

_____. 1966. The bacteriology of Enterobacteriaceae. Copenhagen: Munksgaard.

Kauffmann, F., and P. R. Edwards. 1952. Int. Bull. Bacteriol. Nomen. Tax., **2**, 2.

*CDC Publ., Publication from the Center for Disease Control (formerly the Communicable Disease Center), Atlanta, Ga. 30333.

Chapter 2
Isolation and Preliminary Identification

COLLECTION AND PROCESSING OF SPECIMENS

Isolation of members of the family Enterobacteriaceae from stools, from body fluids or tissues, and from environmental sources, and the differentiation of members of the various genera and species, pose problems encountered frequently by investigators in all bacteriological laboratories. If such work is undertaken it should be done in a systematic manner using acceptable methods; otherwise, not only is the purpose of the investigation defeated, but a false sense of security may be conferred. Failure to detect the typhoid bacillus or failure to diagnose correctly an active case of shigellosis, salmonellosis, or infantile diarrhea caused by a serotype of *E. coli* may have serious consequences. The purpose of this chapter is not to dictate the exact methods and materials each worker should use. On the contrary, the intent is to emphasize the principles involved and to outline acceptable practices that are known to have yielded satisfactory results in many laboratories.

From the standpoint of the effectiveness of the laboratory, nothing is more important than the adequacy and condition of the specimen received for examination. If specimens are not properly collected and handled or are not representative, the laboratory can contribute little or nothing to any investigation. This applies to specimens of all sorts.

FECAL SPECIMENS

Stools should be collected early in the course of enteric disease processes and before institution of treatment. Selected portions should be inoculated onto *adequate* plating and enrichment media as soon as possible after collection, since some etiological agents may decrease rather rapidly in numbers or may be overgrown by other bacteria. When present, pathologic constituents such as mucus and shreds of epithelium should be selected for cultural work. In bacillary dysentery, including the chronic form, immediate plating of swabs taken directly from lesions during proctoscopy is a most satisfactory method for isolation of *Shigella.*

Most competent investigators are agreed that the specimen of choice in enteric disease is a freshly passed stool. Rectal swabs may be used to collect specimens from persons who are acutely ill with diarrheal disease or dysentery, as in hospitals or in the examination of a sampling portion of those ill in a larger epidemic (unpublished data; Hardy et al., 1953). However, ordinary rectal swabs should not be relied upon to yield the maximum number of positive cultures (Shaughnessy et al., 1948; Thomas, 1954; McCall et al., 1966). Further, single rectal swabs are of little value in the examination of convalescent patients or in surveys for carriers. One hastily collected rectal swab, usually an anal swab, placed on a plate of selective medium can hardly be expected to yield numerous colonies of pathogens, especially of shigellae. Yet, this is precisely the procedure used as the basis for many reports recorded in the literature on the incidence of *Shigella* and other enteric bacteria in diarrheal disease.

Whenever possible, repeat specimens should be cultured, i.e., multiple specimens should be examined. Many investigators have demonstrated the value of this procedure. For example, it was demonstrated at least as early as 1916 by Ten Broeck and Norbury. Similar data later were reported by others (e.g., Floyd, 1954). In the examination of suspected carriers, purgation usually results in an increase in the number of positive cultures. This fact has been known for many years in connection with the isolation of *S. typhi* from carriers, for example, and more recently the advantages of purgation again have been demonstrated in the isolation of *Vibrio cholerae* (Gangarosa et al., 1966).

In the investigation of diarrheal diseases, the importance of obtaining stool specimens in the *acute stage* of the disease cannot be overemphasized. Usually the bacillary incitants of enteritis are present in large numbers at that time and often are the predominant organism in the stool. As the symptoms subside, the numbers of the causative agent rapidly decrease so that in cultures taken after the acute stage of the disease is past, the organisms responsible for the infection may be isolated only with difficulty or may not be found.

Transport Solutions And Media. Specimens that cannot be cultured very soon after collection should be placed in a transport solution or medium until they can be examined (or delivered to another laboratory). A

number of transport media have been described; all of them are designed to hold the bacterial population in a specimen more or less stationary, and to prevent, as much as possible, overgrowth of a particular microorganism by others that may be present. One of the oldest known, and probably still the most widely used, is the buffered glycerol saline solution described by Teague and Clurman (1916) and modified by Sachs (1939). The final pH of this solution should be 7.4, and if it becomes acid it should be discarded. Approximately one gram of feces is added to 10 ml of solution and thoroughly emulsified in it.

Several additional methods for transport of stool specimens have been described. Stuart (1956, 1959) devised a semisolid agar menstrum, and advocated the use of charcoal-impregnated, buffer-treated swabs with it. Originally Stuart's medium was designed for transport of specimens from persons suspected of having gonococcal infection, but it has been used successfully in limited studies with stool specimens by Stuart (1956), Cooper (1957), and Ewing et al. (1966). Stuart's transport medium has been modified for various reasons by Cary and Blair (1964), Cary et al., (1965), and by Amies and Douglas (1965) and Amies (1967). These modifications have been used with success for the isolation of salmonellae and shigellae by the above-mentioned investigators and others. These semisolid transport menstra are inoculated with stool specimens collected on buffer-treated swabs, and the swabs are left in the tubes. The method for preparation of the buffer-treated swabs is given in Chapter 18.

Hajna (1955) devised a specimen preservative (SP) solution which in his hands doubled the number of isolations of shigellae and salmonellae when compared with buffered glycerol saline solution. Shipe and Fields (1956) investigated the use of a chelating agent, disodium ethylenediamine tetraacetate (EDTA), in the preservation of coliform bacteria in water samples. Later Shipe et al. (1960) used EDTA in a glycerolated preservative solution with good results. In a small investigation, Ewing et al. (1966) reported the results of comparative studies with 131 stool specimens from as many persons registered as carriers of *S. typhi,* using buffered glycerol saline solution, Stuart's method, and the EDTA glycerol saline solution of Shipe et al. The results of cultures of the fresh specimens, platings from each of the three transport media, and cultures from each transport medium following enrichment in selenite F broth (v. inf.) all were compared. All three transport methods yielded excellent results when cultured directly. About 3 per cent more positive cultures were obtained from specimens in EDTA solution under these conditions, but after specimens in EDTA medium were incubated in selenite F broth, the number of positive cultures was reduced about 65 per cent. This effect of EDTA on selenite medium also was noted by Shipe et al. (1960).

Dold and Ketterer (1944), Lie (1950), and Bailey and Bynoe (1953), among others, reported that drying stool material on pieces of filter or blotting paper was a useful method for shipping specimens. This method may be of value when the use of other methods is impractical or impossible, as might be the case in obtaining specimens from very remote regions.

One other method of transporting specimens, of value only in investigations of outbreaks of salmonellosis, should be mentioned. Swabs (preferably buffer-treated) inoculated with stool specimen, or rectal swabs, are placed in tetrathionate brilliant green enrichment medium (v. inf.) in screw-capped tubes, packed carefully, and mailed. This method is satisfactory for most salmonelleae, but it is of little or no value when *S. typhi, S. cholerae-suis,* or *S. enteritidis* bioserotype Paratyphi-A is involved. McCall et al. (1966) employed this method with success during investigations of an outbreak of salmonellosis in which *S. enteritidis* serotype Derby was the etiological agent.

Methods for preparation and use of all of the transport solutions and media mentioned in the foregoing paragraphs are given in Chapter 18. It is evident that a number of such preservative menstra are available. However, a review of the subject of transport methods (Ewing, 1968) revealed a dearth of comparative data. Comparative studies of the preparations mentioned above by several groups of investigators working under various conditions are needed before an intelligent evaluation of their relative worth can be made. Some of the methods, e.g., Stuart's medium and some modifications thereof, show promise and should be evaluated further. A transport menstrum similar to that devised by Amies and Douglas (1965) and Amies (1967), but with something other than thioglycolate in it, might be a better choice. Such a medium might prove to be better not only for Enterobacteriaceae and allied bacteria, but for other microorganisms as well (e.g., *Bacteriodes*).

Enrichment Media. While it always is advisable to employ enrichment media in the examination of various kinds of specimens, their use is practically essential when dealing with fecal specimens from carriers or suspected carriers. Two such media have been employed extensively and may be recommended for general use. These are tetrathionate medium of Muller (1923) and selenite F broth devised by Leifson (1936). The combined enrichment medium of Kauffmann (1930) is a modification of Muller's medium which contains bile and brilliant green. The efficacy of this