MONTANA STATE UNIVERSITY

A classification according to the mammalian sources of coliform organisms isolated from a remote mountain stream
by Gareth Allen Wilson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
Montana State University
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Abstract:
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Numerical analysis revealed similarity values of similar levels for coliforms from all sources. Only in deer could occasional strains be found that appeared to be unique.

No specific bacterial signatures could be obtained from gas chromatography but statistical analysis of the principle products, acetic and lactic acids, revealed that the organisms obtained from the water were comparable to those from elk (.90P>.50). Coliform strains of human origin were found to be generally distinct from these (P=<.01). The data from the limited number of coliform isolates from deer indicate that these too may be unique.

A CLASSIFICATION ACCORDING TO THE MAMMALIAN SOURCES OF COLIFORM ORGANISMS ISOLATED FROM A REMOTE MOUNTAIN STREAM

by

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A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

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ABSTRACT

Our mountain watersheds are undergoing expanding recreational use. The waters of these streams have been found at times to carry heavy fecal coliform loads. Concomitant recognition of the presence of enteric pathogens make closer examination of these areas for recreational use essential. Indications are that the current techniques for identifying indicator organisms of fecal pollution may be inadequate. It may also be true that fecal organisms may not be the best pollution indicators for the area in question.

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INTRODUCTION

> "To be effective as an indicator bacterium, the indicator must: 1) be present whenever pathogens are present, 2) be present only when the presence of pathogens is an imminent danger, 3) occur in much greater numbers than the pathogen, 4) be more resistant to disinfection and the aqueous environment than the pathogen, 5) grow readily on simple media, 6) yield characteristic and simple reactions ...." Bonde (9).

Until the beginning of the last decade the primary emphasis of sanitary microbiologists was the enumeration of coliform bacteria in public water supplies and what this might mean in terms of potential danger to the community. For this purpose coliforms are defined as "all the aerobic and facultatively anaerobic, gram negative, non spore forming rod shaped bacteria which ferment lactose with gas within 48 hours at 35°C" (2). The water quality standards established by the United States Public Health Service still do not permit the presence of coliforms of any type in water designated for domestic use. The appearance of any microorganisms classed as coliforms is considered evidence of fecal pollution (2).

There are no such restrictions applied to water designated for recreational use. Research (8, 25, 53, 64, 65, 75, 109) has shown that the water of high mountain watersheds are not the ultimate examples of pristine purity they were long considered to be. In fact, high quality water has become difficult to define. Not only are the streams of remote areas found to carry remarkably high coliform

loadings (8, 32, 47, 53, 64, 75, 109), but from bacterial cultures of the water we know that there are enteric pathogens present much of the time (18, 25, 32, 52). Often these areas are used as summer livestock range and certainly the presence of domestic animals can account for many of the Salmonella, Arizona, pathogenic Escherichia coli, Leptospira, and enteric viruses reported. Other investigations (3, 20, 59, 65, 92, 111) have shown that these organisms are also carried endemically in the wild small mammals, bear, and deer populations. Although fish found in remote streams have been implicated as the possible distribution centers of microorganisms, they do not normally carry coliform organisms in their intestinal tract (36, 45, 50).

Most coliforms isolated from water have been found to be of little sanitary significance, being normally found in soil and on vegetation (32, 33, 37, 39, 75). Still other types are associated with the feces of warm blooded animals (34, 70). The latter are the types normally associated with the presence of pathogens. Most recent research has dealt with methods for distinguishing between the two types of coliforms. While some degree of success has been achieved in distinguishing the organisms of no sanitary importance from those that are, no good method yet exists for differentiating organisms obtained from human and other mammalian fecal sources (33, 34, 47).

In view of the numerous occasions in which deer, smaller wild animals, and domestic livestock have been found to be carriers of enteric diseases (25, 65), a means of distinguishing between the coliform organisms originating from their respective body discharges may be superfluous. Rather, any water identified as containing fecal contamination should be considered to be polluted water (32). On the other hand, there is a shortage in the literature to date of references implicating big game such as elk and moose as carriers of enteric disease. If this is true, and since it is difficult to cure many streams of their fecal pollution, being able to distinguish the type and source of pollution would simplify a decision concerning the suitability of a body of water for use. Development of techniques for isolation and identification of coliforms according to the species of animal from which they derive would also make epidemiological studies simpler and facilitate removing a source of pollution.

A review of the coliform types and the means by which those of sanitary significance are separated from those that are not is in order. Also to be included is a short survey of other organisms associated with fecal material and why these are not, as yet, considered to be of use in sanitary procedures.

Identification of Coliform Bacteria

Coliforms have already been described as gram negative, lactose fermenting (generally), aerobic or facultatively anaerobic rods. For sanitary purposes, they have traditionally been identified as a group by using a battery of tests known as the presumptive (ability to produce gas in lactose broth at 35°C in 48 hours), confirmed (can produce gas in brilliant green bile broth in 48 hours), and the completed tests (growth in selective media and morphological identification) as outlined in Standard Methods for the Examination of Water and Wastewater (2).

To this battery has been added another technique, the membrane filter. This procedure allows the identification of colonies demonstrating a metallic sheen over all or any part of its surface as coliforms while at the same time allowing an estimate of the numbers present to be made (2).

For more selective identification there are many suggested biochemical procedures in the literature taking advantage of the action of the organism on peptones, sugars, and alcohols, as well as their ability to grow at elevated temperatures (19, 22, 24, 34, 36, 66, 96). From this large group of tests, two have been selected as giving the most information in the shortest possible time, for routine use in identifying fecal coliforms (2). The best of these, the Eijkman fecal

coliform test, is based on the finding by Eijkman (51) that members of the Escherichia group could cause the production of gas in a glucose medium at 46°C whereas organisms of the Enterobacter (Aerobacter) group could not. The test was modified by Hajna and Perry (50, 86) to include bile, a buffering system, and the substitution of lactose for glucose in the medium. The medium with these modifications constitutes the EC (E. coli) broth recommended for performance of the test (2, 50, 86). A final modification (35, 37) lowered the temperature of incubation to 44.5°C ± 0.2°C for 24 hours in a water bath. This is the procedure now recommended for routine use in distinguishing fecal from non-fecal coliforms (2). The principle of the test takes advantage of the fact that fecal coliforms produce the enzyme, formic hydrogenlyase, at 44.5°C reducing formic acid formed during lactose fermentation to hydrogen gas and carbon dioxide (19).

Also recommended, but much less specific in identifying the fecal origin of the coliforms, is the indol, methyl red, Voges-Proskeuer, and Simmons' citrate (with the mnemonic IMViC) group of tests. Of these tests, the methyl red test is of greatest value (32). Data demonstrating its usefulness in identifying fecal coliforms will be cited as a matter of interest. The IMViC results are expressed in a formula of plus (+) and minus (-) signs to describe the difference

between organisms. By use of the formula sixteen strains, ranging from ++++ through ++-- and --++ to ----, all of which have been found, are possible. The IMViC test series can be used separately or in conjunction with the EC test in an attempt to identify sources of fecal pollution. For this purpose the most important types will be described.

The groups ++-- (E. coli var. I), -+-- (E. coli var. II), and +--- (uncommon) are considered to be Escherichia species (2) and are the most frequent indicators of fecal pollution since they are found to occur universally in the gut of nearly all warm blooded animals in all sections of the world (2, 10, 33, 34, 103). These strains contribute the majority of positive fecal coliform (EC) tests, roughly 90% depending on the author, and are referred to as fecal coliforms (34, 39, 70). It must be noted that type -+-- is EC positive far less frequently than ++--, 22% and 93% respectively, again depending on the author (34, 70). A common complaint concerning the use of the ++-- type as fecal indicator is its relatively lower survival rate in an aquatic environment (30). Among the pathogens, -+-- is also the type attributed to Shigella sp. (10, 22) and accounts for the Shigella-like organisms reported in pollution surveys (47).

Types -+-+ and ++-+ are considered to be Escherichia (Citrobacter) freundii types I and II respectively (2), and are principally

of non-fecal origin (10). Strains with the formula --++ (Enterobacter aerogenes var. I), --+- (E. aerogenes var. II), and ---+ constitute the Enterobacter group (2). For reasons to be discussed the enterobacters cause more confusion in the interpretation of fecal pollution than does any other type.

The widest diversification of IMViC types is found in human stools; eleven have been found with the following six predominating: ++--, -+--, --++, -+-+, ++-+, and +++- (34). The ++-- group occurs 87.5% of the time with --++ next most common at 5.4%. Mishra et al. (70) conducted a survey in India and reported that 52.4% of the Enterobacter group --++ responded positively to the EC test. In all, the EC procedure correctly identified 96.4% of all colonies isolated as being of fecal origin. E. coli var. I (++--) type, of course, gives maximum correlation with EC positive tests (34, 38, 70). It is interesting that the methyl red test successfully identified 94.7% of all fecal strains (33).

From the droppings of livestock and other animals, Geldreich et al. (33, 34) isolated six coliform types with the following three predominating: ++--, +++-, and ++-+. Of these 96.2% were identified by the EC test and 99.9% by the methyl red test. From these non-human sources the ++-- type again predominated (95.6% in animals, 97.9% in poultry). The strain --++ was found in 3.3% of animal

droppings surveyed in this country (34) but comprised 12.3% of all organisms found by Mishra and his group (70) in India.

In unpolluted soils the prevailing types are -+-+ (48.0% - 52.0% vs. 0.5% - 1.1% in feces) and --++ (18.8%) (33, 37). Correlating results of EC tests are usually negative and showed fecal coliforms to be usually absent or at a low level in undisturbed soils. The numbers of bacteria per gram of unpolluted soil are also low (28). The number of types of coliforms as well as the number of organisms per gram rises sharply with the degree of pollution in disturbed soils along with the number of positive confirming elevated temperature tests (37).

A similar situation exists on plants, where the types --++ and -+-+ are also found but the type ++++ (24.2%) predominates (39). The type ++-- was found to be present nearly 11% of the time and usually was confirmed by means of the EC test as showing the presence of fecal pollution (39).

It is the soil microorganisms that constitute the principle source of stream contamination during surface runoff (64, 75, 114). The types that are isolated from the waters of many streams correspond most readily to the types found in the soil with few of the types indicative of those found on vegetation being present (33). Fecal coliform numbers in polluted water rise in direct proportion to the amount of

soil contamination by animal wastes. This is true not only in those areas put to use by man for himself and his animals, but also in the most remote high mountain streams draining watersheds extensively used by wild animals (8, 25, 47). Goodrich et al. (47) have demonstrated a high percentage of positive fecal coliform tests performed on microorganisms isolated from a closed mountain watershed used almost exclusively by big game. Research conducted simultaneously on an adjacent watershed open to recreation where big game did not congregate actually demonstrated a lower level of coliform organisms (109). The land area in the closed watershed was extensively covered by big game and some horse droppings, from which debris continuously washed into the stream during surface runoff.

That better methods are required in distinguishing fecal coliforms is demonstrated by the number of IMViC type --++ (Enterobacter sp.) giving positive reactions to the elevated temperature test. This group has commonly been reported isolated from stool specimens as well as from soil. Mishra et al. (70) found that 52.0% of Enterobacter types found in human feces gave positive elevated temperature tests while 43.6% from soil also gave positive reactions. Hendricks (51) reports finding positive EC reactions with a number of Enterobacter isolated from river water in Georgia. Since he compares these with an EC negative "wild type" he obtained from the American

Type Culture Collection, supposedly isolated from a fecal source, he maintains that those EC positive Enterobacter found in the "natural environment" are of non-fecal origin. Dufour and Cabelli (21), in a study comparing EC results to assigned designations of organisms identified by other means, identify the EC negative Enterobacter (96.0% of the organisms in this study) to be of non-fecal origin, while the EC positive organisms overwhelmingly fit the description of Klebsiella and are of fecal origin.

Also to be considered are the 2.0-6.0% of ++-- coliforms of fecal origin that do not give positive fecal coliform tests. This could mean that an equal percentage of water in which they are found could be declared unpolluted when such is not the case (70).

For the reasons discussed, the separation of coliforms into fecal and non-fecal sources is inadequate when based on the IMViC tests which are time consuming and require pure cultures to perform. Also, a not insignificant number of fecal coliform negative samples from stool cultures using the EC test make this test somewhat unreliable as well.

As yet, then, there is no completely satisfactory method available for providing reliable information for determining whether an organism is of sanitary significance let alone any idea of its mammalian origin. However, significant progress has been made in this

direction using several techniques.

E. coli serotyping has been successfully used to identify these microorganisms among those found contaminating streams. By isolating the same types at several sampling points along a water course, the probable source of pollution has been identified (8, 44, 45, 87). There are disadvantages here as well. The majority of organisms isolated that are successfully typed do not appear often enough in a particular study for relationships to be established. The E. coli typing sera used are those prepared commercially against largely human strains of the bacteria and consequently, at times, have little antigenic relationship to those found in the environment. A study by Bissonnette et al. (8), using commercial antisera, found common serotypes of E. coli in both water and fecal samples from big game. Cross reactions among antisera with the bacteria were common and a great many more (approximately 50.0%) did not agglutinate at all. Glantz (43) found that many E. coli strains believed associated with livestock diseases were untypable with commonly used commercial typing sera. By producing antisera against these untyped strains, he was able to establish serologic relationships to disease-causing organisms as well as finding the same organisms in polluted water.

A second serological procedure successfully tried is the use of fluorescent antibody staining of the colonies on a membrane filter.

This technique provided specific identification of the colonies fluorescing as the result of contact with known antisera as well as a method of enumerating the bacteria present since known dilutions of the water sample could be used and multiplied by the number of fluorescing colonies seen on the filter. The colonies are counted with a dissecting microscope and ultraviolet illumination (49).

Serological procedures in general have the advantage of high specificity but preparation of the antisera used has the disadvantage of requiring large areas for rabbit maintenance in addition to the amount of laboratory equipment needed to accomplish the serological procedures themselves. In research projects in which large numbers of microbial strains have been isolated, the outlay in space, equipment, and personnel is great indeed in order to adequately survey even a single watershed.

A relatively simple tool that has been put into use since 1963 (1) in the taxonomic identification of bacterial strains is gas chromatography. This method takes advantage of the fact that lipids, carbohydrates, amines, and metabolic by-products of the bacterial species are different to a degree and have been successfully used in identifying specific microorganisms in a number of instances. Since this method is important to the completion of this research project, it will be separately described below.

Fecal Streptococci as Pollution Indicators

As has been pointed out, the use of coliform bacteria as indicators of pollution has many shortcomings. Most coliforms have been shown to originate from plants and soil and are found to be innocuous in nature. Those that are regarded as positive identifiers of fecal pollution are found inconsistently in polluted waters. Worse, the ++-- type confirmed to be the principal fecal coliform has been discovered to have a shorter survival time in an aquatic environment than many pathogens. At times then, pathogens may go undetected when tests for fecal coliforms are negative (31, 52, 105). The type regarded as representing non-fecal pollutants, --++, is rather frequently found in fecal sources and give every positive reaction of fecal microorganisms. The possible differences in the genera of this type have been described but are far too complicated for the average public health personnel to give them every day consideration.

Large numbers of other genera are found in the gut of animals and many of these have been examined in the attempt to better define fecal pollution. Of these, the streptococci, are found to occur in the droppings of all warm blooded animals and are consistently isolated from the water receiving their discharges (2, 40). In cases where the water contamination was known to be recent or continuing, streptococcal counts, when considered simultaneously with fecal

coliform data derived from the same water, could be used to identify the source of polluting discharges (2, 32, 40). Fecal streptococci are found to be present in similar or greater concentrations than coliforms in fecal discharges of farm animals, other domestic animals, and various wild game. In humans, coliforms occur in numbers four times greater than do the streptococci (64). Also, studies have found that Streptococcus bovis and S. equinus are isolated only from warm-blooded, non-human animal populations, which is a particularly useful characteristic in defining pollution sources (32, 40, 58).

It was also noted that fecal streptococcus numbers increased greatly in storm water runoff. This increase is due to the presence of S. faecalis and S. faecalis var. liquifaciens (40). These organisms are ubiquitous in the environment and are of no value as pollution indicators; these organisms are rarely found in feces of warm blooded animals. These strains show greater survival (13, 32) in an aqueous environment than those from the alimentary tracts of warm blooded animals. Membrane filter cultures from streams heavily contaminated with these strains mask the growth of the known fecal strains. Such large numbers of common streptococci make identification of smaller numbers of significant fecal streptococci difficult even if the latter are present. This fact makes interpretation of results from fecal streptococcus testing programs difficult and risky,

thus making the use of streptococci as fecal identifiers of limited value (32, 40).

Other Microorganisms as Pollution Indicators

A discussion of the difficulties of specific pollution identification using fecal microorganisms must include the preference stated by some for growing the pathogens directly while ignoring the conventional organisms already discussed (2, 31). No authority, including Standard Methods (2) recommends the use of this procedure in lieu of those methods known to be effective for coliforms and streptococci; but in certain situations such findings are definitely significant and methods and media have been developed whereby this can be readily accomplished (2, 52, 104).

Arguments in favor of such procedures center around the apparent occasional ineffectiveness of the standard procedures for the detection of pathogens (31). Fecal coliform counts may be low or non-existent when salmonellae may be at a maximum (48, 95). Research has shown that some salmonellae and possibly even some shigellae (31, 81, 111) have a greater survival rate under adverse conditions than does E. coli. Generally, however, it has been shown that a minimal number of fecal coliforms must be present for infectious levels of pathogens to occur (25). Also, the release of the pathogens themselves are known to be so sporadic that an outbreak would likely have

occurred by the time the organisms could be grown in the laboratory. For this reason a constant check of the level of fecal coliforms is much preferred.

More practically, in Europe, especially, the use of other organisms occurring in the intestinal tract has been considered as fecal pollution indicators (9). Foremost among these are the sulfite-reducing anaerobes such as Clostridium perfringens. Not only is their survival much better than that of E. coli, sulfite reducers are a much more homogenous group than coliforms and do not multiply in the soil. They can be used to estimate the recentness and extent of an instance of pollution, their numbers varying in direct relation to the distance from the source of pollution (9). Objections to the method are based on the ubiquitousness of C. perfringens (115) and other sulfite reducing spore formers - i.e., C. sporogenes - in the environment. However, C. perfringens is the principal sulfite reducing organism found in the majority of pollution cases (9). Significant increases in numbers of these organisms over those originally present can be easily determined by establishing background counts (or "background pollution") for these organisms in soil and water (9).

Two remaining organisms also have received consideration as pollution indicators. Staphylococcus aureus is normally found on human skin and mucous membranes and is more resistant to halogen

disinfectants than coliforms. Pseudomonas aeruginosa is found in human otitis externa and other infections. Demonstration of the absence of either of these organisms would be of immediate value in determining the safety of recreational waters, especially those used for swimming (2).

It is obvious that a variety of methods is being recommended for identifying specifically fecal pollution, other than identifying fecal coliforms. Such a method may be readily established by use of the system discussed in the next section.

Gas Chromatography

In the 20 years since James and Martin (61) first reported a successful separation by gas-liquid chromatography many applications have been discovered for it. Continuing improvements in gas chromatography (GC) columns and detector systems have made possible increasingly sensitive determinations. It has proven a rapid, reliable, sensitive, qualitative method of separating complex mixtures of volatile compounds. Any mixture of volatile compounds or ones that may be rendered volatile by the simple methods now available (29, 54, 77, 80, 87, 110) may be successfully separated on a GC column at a cost that need be no more than that of a research microscope. Examples of its use are the ease and reliability with which pesticide studies may be made in environmental suitability surveys of suspect watersheds

or the elucidation of the fatty acids in butterfat. In pre-GC days, butterfat was thought to contain 11 fatty acids; today over 140 components are known (54).

Abel, DeSchmertzing, and Peterson (1) in 1962 first successfully applied this system to microbiology. On the theory that differences in chemical composition of bacteria would be governed by natural evolutionary relationships if the organisms were grown under defined conditions, they methylated and analyzed cell lipids. They were unable to distinguish between genera of Enterobacteriaceae using the method but were able to find differences in the lipid profiles of selected families of the class Schizomycetes.

Since this primary effort, gas-liquid chromatography (GLC) has been applied not only to distinguishing species and strains of bacteria in pure culture (72, 73, 76, 79, 80, 90), but also when their presence has been suspected in pathological situations (27, 74).

Most chemical compounds, lipids, carbohydrates, and amines found in a bacterial cell have now been volatilized (28, 29, 78) and the data obtained have provided much information in clarifying taxonomic relationships among microorganisms. Several major analytical approaches have been used. The first of these is the examination of pyrolyzed products of whole bacterial cells. This is a complicated procedure because of the difficulty encountered in standardizing the pyrolysis

of whole cells due to the critical nature of time and temperature, composition of the medium used for growing the cells, and age of cells (16). A commonly used procedure is the extraction of specific chemical compounds directly from the cells. These compounds are then volatilized (28, 29, 80) and the resultant chromatograms examined for the presence or absence of designated peaks, i.e., "signatures" or patterns characteristic of a given organism. Even if the peaks obtained are all similar, a situation often encountered in distinguishing between the strains of a species, the peak area may be calculated and strain differences may often be noted statistically (54, 87).

A method used as widely as the extraction and volatilization of specific chemical compounds from the cells, is the use of media extracts (54, 72, 83, 110) or head space gas (106) from metabolic products synthesized by the organisms during growth. This is probably the simplest of all the recommended procedures (54, 74, 93, 110); no special techniques are required, the products are easily extracted and injected directly into the machine. All bacteria synthesize many volatile compounds during their growth cycles. Those compounds present as non-volatile metabolic products may be easily converted by a simple methylation procedure (110). Together they provide valuable information concerning the identification of bacteria.

Bowdon and Basset were the first to distinguish cultures of E. coli and E. aerogenes using head space analysis (106) of the volatile metabolic products of these organisms. More sensitive detectors have since made the detection of specific types of bacterial growth possible at very early stages (71, 72, 73, 74). The only limits on the procedure would seem to be the sensitivity of the column and detector.

Columns adequate for determining all the volatile compounds of an organism are difficulty to achieve. The metabolic products, volatile and non-volatile, are polar compounds and by the use of a corresponding polar stationary phase column, most difficulties may be overcome and a reasonably broad range of elutions may be expected (69).

The choice of a detector is somewhat simpler. Flame ionization (FID) is the most popular method giving good response with a broad range of compounds (69). It also has the advantage of being water insensitive thus making extraction procedures easier. It is also insensitive to formic acid during the analysis of volatile lipids (those lipids containing 1-8 carbon atoms).

The electron capture detector (ECD), extremely sensitive in the nano and pico gram range, has been used in highly sophisticated procedures, often in combination with FID, for early detection and identification of microbial growth. It is quite expensive and has

the added liability of being virtually insensitive to alcohols, common by-products of microbial metabolism (69).

The thermal conductivity detector (TCD) has also been used in a number of procedures; it detects formic acid readily (55, 62, 93, 104) and, at a temperature of 125°C, recommended for the analysis of C_{1-8} volatile fatty acids, is thought to have a greater sensitivity for these compounds (12). It is generally less sensitive than FID or ECD and has the added disadvantage of trailing water peaks at the beginning of a chromatogram. This requires careful extraction from the parent solution of the compound to be volatilized (110). It has proven quite useful for simple, low cost identification of the anaerobes which produce a variety of volatile and non-volatile acids as a result of their metabolism (110).

A range of volatile fatty acids and non-volatile compounds similar to those found in the method recommended for anaerobe identification using a TC detector is found in the mixed acid fermentation of the methyl red-Voges Proskauer tests of the IMViC series. Fermentation of glucose by *E. coli* for the methyl red test is typified by a high yield of acetic, lactic, formic, succinic acids, and ethyl alcohol. The aerogenes type of fermentation, the basis of the Voges-Proskauer test, is typified by the formation of neutral products such as 2,3-butanediol and ethyl alcohol; other volatile and non-volatile compounds are

present in lesser amounts (19, 102).

It would be a simple procedure to adopt the methods of anaerobic microbial identification in an attempt to elucidate differences in strains of coliforms producing these products. Before attempting such a procedure, a thorough analysis using as many biochemical tests as practical should be carried out in order to group the organisms as nearly as possible. The source of a strain, whether from water, soil, or the droppings of a particular species of livestock or wild game should receive primary consideration. The use of similarity values (88, 97, 101) from numerical taxonomy would prove useful in placing strains in groups from which organisms could be selected for determining the relation of the group to other groups or even an individual organism if these prove physiologically distinct.

Statement of Experimental Purpose

The work by Stuart et al. (personal communication) on the Mystic watershed above Bozeman, Montana yielded a collection of a number of pure strains of coliform bacteria. The organisms were isolated from the stream water and from the feces of elk, deer, bear, and horses deposited on the ground drained by the stream.

As many of the coliform cultures as possible were selected for full biochemical evaluation followed by grouping of those strains with the same reactions. From these, strains were selected for

further study in a gas chromatograph using a thermal conductivity detector. It was expected on the basis of the components of the mixed acid reaction that the chromatograms would show similar peaks. Determination of strain differences were done by measuring the relative areas of the peaks formed and their statistical comparison to peak areas from other groups of organisms.

Testing emphasis was primarily on those organisms of IMViC formula -+-- and ++-- with accompanying positive EC tests since these are defined as fecal coliforms. Other IMViC types were included for comparative purposes.

MATERIALS AND METHODS

Selection of the Test Microorganisms

Selection of coliforms for further testing from those isolated by Stuart and his group was based on the following criteria: (1) the remoteness of the region from which an organism had originally been isolated and (2) the reactions originally shown on EMB agar, the IMViC group, the EC test, and lactose fermentation.

To ensure the organisms selected were indeed from regions with minimal contamination from humans or their livestock, selections were made on the basis of the map of the area and the location (8) from which the organisms had been isolated. Only coliforms from the most remote sections showing few indications of use other than by wild game were chosen. Since the site was also used to a limited extent by horses, droppings of this species were included in the study group to discover their relationship to isolates from wild game.

Organisms were selected for study so that large numbers of fecal strains would be included. It may be seen in Appendix A that well over half of all strains chosen fit the description of fecal organisms as explained in the discussion. EC tests had been done on only those organisms isolated from water. Fecal coliforms from animal droppings were selected on the basis of a metallic green reaction on EMB, typical of *E. coli* (2), and the IMViC patterns. EC test results were also considered in the selection of water strains. Strains typical of

E. coli var. I constituted over one half of those chosen. Isolates resembling E. coli var. II (-+--) and large numbers of IMViC types -+-+ and --++ were included to discover the relationship, if any, of these other common types to fecal flora. Coliforms isolated from deer, in particular, lacked the typical coliform pattern, showing predominantly types -+-+ and --++. As a matter of interest, small numbers of other types were included in rough approximation to the proportion in which they appeared to occur in the original tests.

Also selected for identification were strains showing typical Salmonella-like or Shigella-like reactions on MacConkey agar, in the IMViC tests, and on Kligler's iron agar (KIA) when the latter had been done. This was done to determine if there were any pathogens being washed into the stream along with the coliforms.

The number, types, and sources of the strains ultimately chosen are listed in Table XIV. Those with laboratory numbers beginning with 2 are from water; those beginning with 6 from wild game or horses. The letter following the number designates the species from which it was isolated (see Appendix A key). Strains from elk ultimately predominated among those selected because the area is one inhabited by an elk herd. Horses were next most numerous followed by deer and bear. The moose sample was included for interest.

Ten E. coli strains isolated from human stool samples were received from the Montana Department of Health Laboratory in Helena. These were handled in the same manner as the other organisms for later comparison by numerical taxonomic methods and in the gas chromatograph.

All organisms were reisolated on EMB and MacConkey agar to ensure continued viability of the organisms and purity of the strains. Individual colonies were inoculated to fresh stock culture slants, incubated for 24 hours, then stored at 4°C until use. Those cultures chosen that could not be reisolated in this way from the original stock culture slants were heavily inoculated into trypticase soy with 2.0% yeast extract broth (TSY) and reincubated. If after 48 hours, no growth appeared, 5.0 ml of broth was placed in the culture tube and the tube incubated for seven days with transfers to fresh TSY broth at two day intervals. If at the end of the designated time, no growth appeared, another culture was selected from the same general group.

Control Microorganisms

To be certain that the media used in the biochemical investigations were reacting properly and to provide a source of known reactions by various organisms for comparison purposes, a number of known strains of bacteria were used.

On hand at Montana State University were strains of Escherichia coli, Enterobacter (Aerobacter) aerogenes, Shigella flexneri, Salmonella typhosa, Enterobacter cloacae, Enterobacter alvei, Proteus rettgeri, Providencia alcalifaciens, Salmonella enteriditis bioserotype D, Salmonella paratyphi B (S. enteriditis bioserotype paratyphi B), Shigella dysentariae, and Arizona sp.. From the Montana State Department of Health Laboratory in Helena were obtained Flavobacterium sp., Alcaligenes denitrificans, Herbicola-lathyri group, Edwardsiella tarda, Aeromonas hydrophyla, Pseudomonas stutzeri, Pseudomonas diminuta, Pseudomonas putrifaciens, Alcaligenes faecalis, Proteus morganii, Mima polymorpha, Shigella sonnei, Proteus mirabilis and an indole-negative Escherichia coli.

Classification

Classification of Enterobacteriaceae was based on that of Ewing (23, 66, 96). This system appears to supersede that found in the current edition of Bergey's Manual (10) according to a majority of modern texts (66, 96) and papers (24, 116). For the non-enteric types isolated, Bergey's Manual (10) and Hugh (57) served as references.

Preliminary identification of organisms was by means of the Elizabeth King (Bartlett, personal communication) classification system for gram negative microorganisms. The procedure takes advantage of the fermentation of 1% glucose by Enterobacteriaceae in

the oxidation-fermentation (OF) medium of Hugh and Leifson (57) as well as their oxidase negative characteristic in Kovac's (63) oxidase reaction. These reactions were used to separate the Enterobacteriaceae from the other gram negative bacilli.

Following preliminary separation by use of the King system, subsequent identification of all strains was done by a battery of tests selected from Edwards and Ewing (24), Ewing (22), and Martin (66).

Culture Media and Reagents

The tests, media, and references to procedures employed are as follows: Levines EMB agar (BBL), MacConkey agar (Difco), Kligler's iron agar (KIA, Difco) were prepared as directed (7, 85, 108), growth and reaction on each of these were recorded after 24 hours incubation. In the event of clear colorless (non-lactose fermenting) colonies on MacConkey agar, the plates were held for another 3 days to watch for slow lactose fermenting organisms and consequent changes in the indicator. All growth observed on the media and reactions during the biochemical tests were recorded as shown in the key to Appendix A.

For the OF reaction, two 13 x 100 ml tubes containing 3 ml of OF basal medium (Difco) with 1% glucose added were lightly inoculated and one overlaid with about 5 mm (0.5 ml) of sterile paraffin oil. The tubes were then incubated and observed daily for up to four days for

acid formation (57, 108). Acid formation in both the open and sealed tubes was recorded as a fermentative reaction, acid formation in only the open tube was recorded as an oxidative reaction, and failure to form acid in either tube indicates failure of the organism to utilize dextrose by either method.

The oxidase test was modified as directed by Paik (84). For this, 1% dimethyl-p-phenylenediamine was prepared fresh and a drop placed on the surface of the culture plate. Color changes of pink-maroon-black, usually seen within five minutes, indicated a positive reaction.

Indole tests involved incubating 5 ml of inoculated peptone water (1.5% tryptose in water) for 48 hours. One-half ml of Kovac's reagent (84) was added to the culture followed by gently shaking the tube. A deep red color collecting at the surface of the broth was considered positive.

For the methyl red-Voges Proskauer test, two tubes containing 5 ml of MR-VP broth (Difco) were inoculated and incubated for 48 hours. After this period, 2 ml of modified O'Meara's reagent (for the detection of acetoin) (84) was added to one of the tubes, the tube shaken and reincubated for 4 hours with hourly observation. A positive Voges-Proskauer test was indicated by the development of a red band at the top of the broth culture. If results were difficult

to read, the test was repeated with incubation of the culture at room temperature.

The second tube, for the methyl red test, was reincubated for 2 more days. This four day incubation period was suggested by Paik (84). After this time, four drops of methyl red indicator (84) was added to the tube. A red color was formed if the test was positive.

MR-VP broth used for the analysis of metabolic products by gas chromatography was prepared as for the MR-VP test, then tubed in 10 ml amounts in 18 x 142 mm anaerobic culture tubes (Bellco #1803-S1). Further treatment of this medium will be discussed under gas chromatography.

Simmons' citrate agar (BBL) was prepared and incubated as directed. A blue coloration of the medium after 24 hours or more was taken as positive. The tubes, if negative, were observed for seven days since this is the time indicated for some Providencia strains to react (7).

Urea broth (Difco), motility agar (Difco), KCN broth base (Difco) with 15 ml of 0.5% KCN solution added, phenylalanine agar (Difco), gelatin medium (Difco) were all prepared and used according to directions. The KCN broth must be cold when the KCN solution is added. The 10% ferric chloride used in the phenylalanine reaction was prepared only once and stored at room temperature. Reactions

were recorded as described in Appendix A.

The Falkow (26) decarboxylase broth was prepared in four portions. To two portions 0.5% lysine and arginine, respectively, were added; to a third 1% DL-ornithine (the pH of this portion usually required readjusting to 6.8) (66). The fourth portion of the base was retained as a control and contained no amino acids. For each organism a set of 4 tubes containing 4 ml of the respective broths was inoculated and overlaid with about 4 mm (0.7 ml) of sterile mineral oil. Non positive tubes were allowed to incubate for up to 4 days. Reactions were recorded as negative if only an acid reaction resulted. Positive reactions were recorded for alkaline (purple) reactions. If the indicator in the control tube remained unchanged (blue) the organisms did not ferment glucose and the proper conditions for the reaction could not be obtained (21). These too were recorded as negative.

Organic acid media and purple broth base were both prepared from individual reagents as directed by Vera and Dumoff (108). One percent mucic (galactaric) acid was added to the organic acid broth base and boiled until the acid dissolved. The pH was readjusted to 7.4 with 10% NaOH and 4 ml of the medium dispensed into 13 x 100 mm test tubes and autoclaved. A lemon yellow (acid) reaction in the medium, usually occurring within 1-4 days, was recorded as positive.

The tubes were incubated for a total of 15 days as recommended.

Purple broth base was used as the base for the carbohydrate fermentation reactions. One percent sucrose, glucose, lactose, and mannitol or 0.5% dulcitol, salicin, adonitol, or inositol (22) were used. All except sucrose and lactose were weighed and placed directly in the flasks at the time the media were made. The broth was dispensed in 4 ml amounts to test tubes, 50 mm durham tubes added, the tubes capped and autoclaved. Sucrose and lactose were prepared as 10% solutions, filtered through a millipore membrane filter, and dispensed in 0.4 ml amounts to tubes containing 4 ml of autoclaved broth base, giving a final lactose and sucrose concentration of about 1%. Uninoculated controls as well as controls containing organisms known to utilize each respective sugar were run with each batch of test sugars. The cultures were incubated for up to four days and the time of carbohydrate utilization by the organisms recorded.

EC medium (Difco), prepared and used as recommended in Standard Methods (2) was incubated after inoculation from a 24 hour TSY broth culture at 44.5°C in a water bath with gentle shaking for 24 hours. A positive reaction was recorded if gas was formed at the end of that time.

Trypticase soy broth (Difco), used for providing viable cultures for the EC test and inoculating the MR-VP broth employed in the gas

chromatographic analysis, was prepared as directed. As already described it was used with 2% yeast extract added in the attempt to recover non-viable cultures stored on stock culture slants.

All incubation temperatures, except those of the EC test and gelatin liquification tests (room temperature), were done at 37°C.

With the following exceptions 16 x 125 mm tubes (Kimble-Kim Pak) with Morton closure type caps were used for all media; stock culture agar and Kligler's iron agar were tubed in 17 x 150 mm screw cap test tubes; gelatin, OF medium, KCN broth and organic acid media were tubed in 13 x 100 ml tubes. MacConkey and EMB agars were placed in 15 x 100 mm plastic petri dishes.

For use with known control cultures of Salmonella and Shigella and against the few unknown organisms isolated where serological examinations appeared necessary, the following Difco antisera were used: Salmonella-O-antisera-groups A, B, C_1, C_2, D, E and poly A-I, Salmonella anti-Vi, Shigella antisera poly A, B, C, and D. Anti-Escherichia coli sera were not employed as many of these had already been surveyed by Bissonnette (8). The procedure employed for serotyping involved suspending a clump of cells taken from a stock culture slant with a wire loop in a drop of 0.85% saline on a slide. A drop of antiserum was then placed next to the saline suspension of organisms and the two mixed by tilting throughout the procedure.

Strong clumping of the cells within three minutes was regarded as positive. Suspected Shigella cultures that failed to clump were suspended in distilled water and boiled for half an hour. The test was then repeated. If clumping still did not occur, it was regarded as negative.

Serological typing was done only if the following criteria applied: (1) colonial morphology on MacConkey agar (if clear, color-less colonies were found); (2) appearance of KIA (23, 24, 66); (3) urea reactions (urease negative); and (4) the phenylalanine deaminase test (negative). Despite the appearance of any other reactions no further testing was done if one or both of the urea or phenylalanine tests were positive (22).

Numerical Taxonomy

All organisms were tested in the entire battery of tests shown in Appendix A. Upon completion of all the tests, the bacteria were placed in major groups according to their IMViC reactions. These were then broken into subgroups according to the similarity of the rest of their reactions. Strains from water and animals were grouped together on the basis of their colonial morphology and biochemical reactions in order to show as nearly as possible their relationship to each other.

Following completion of this preliminary grouping, the cultures were subjected to a numerical analysis to determine the similarity

values of the isolates (4, 88, 101). Due to time and the number of groups involved, only certain of the strains were given this treatment. None of the IMViC types with smaller numbers were analyzed, whereas all of the -+--, -+-+, and --++ types had just sufficient groups that all could be handled in the analysis. From the ++-- type, with type, with well over a hundred possible groups and single strains, 53 organisms and strains were selected for treatment. In addition to testing the closeness of relationships between groups, strains from water and mammals placed in the same group by biochemical reactions, were tested to determine the closeness of fit of this type of grouping. The numbers assigned to each group or strain and the culture numbers of the organisms the number represents are listed in Appendix B.

The principles of numerical classification (88, 97) require that as many characteristics as possible be determined, each of which is usually given equal weight. At the same time all characteristics held in common by all the organisms, or characters used to define groups, such as IMViC reactions in this case, should be omitted. These restrictions left only 20-22 characters for the -+--, ++--, and -+-+ types to be compared between each of the groups and only 17 characters were considered significant for the --++ type. This is really quite a small number for comparison purposes. In Table I are

Table I. Characteristics employed in numerical analysis.

Test	Reactions considered as positive (1 point)
A. Standard 20 tests used for analysis.	
Growth on MacConkey agar	Pink colonies (for --++ pink is (0)).
Growth on EMB	Metallic green (for --++ metallic green is (0)).
KIA	Acid reaction on slant.
Gas	Presence of any on KIA or in carbohydrate media.
EC	Any gas at 44.5°C (not considered with --++).
Motility	Presence of any noticeable clouding.
Gelatin	Liquification
Lysine decarboxylase	Purpling of medium.
Arginine dihydrolase	Purpling of medium.
Ornithine decarboxylase	Purpling of medium.
Mucic Acid	Yellowing of medium (acid).
KCN	Any clouding of the broth.
Utilization of lactose, sucrose, salicin, inositol, adonitol, esculin	Acid reaction (salicin and adonitol omitted from consideration with --++).
(+) Decarboxylase	Decarboxylase test positive in greater than 48 hours.
(+) Carbohydrates	Carbohydrate test positive in greater than 48 hours.
B. Tests considered for certain types only.	
H_2S	Presence of any (used for -+-+).
Phenylalanine	Greening of the medium (used with -+-+).
Urea	Presence of any urease activity (used with --++).

listed the characters used to define the similarity values of the various coliform strains with the reactions added to or deleted for each noted.

The similarity of each strain was determined by the equation developed by Sokal and Michener (101).

$$\text{Similarity value (S\%)} = \frac{NS}{NS + ND} \times 100$$

NS = Number of characteristics scored positive or negative for both strains.

ND = Number of characteristics scored positive for one strain and negative for the other.

Positive characteristics were scored as one (1), negative characters as zero (0). The similar and dissimilar values were then totalled and inserted in the formula. Each group of an IMViC type was compared to all the other groups of the same type. The values were then placed in a triangular matrix for comparison. No attempt was made to arrange the values in the matrix in numerical order.

Gas Chromatography

The procedure for gas chromatography is taken almost entirely from the methods recommended for the identification of anaerobes by gas chromatography found in the Virginia Polytechnic Institute Anaerobe Laboratory Manual (110). Procedures used will be derived from this manual unless otherwise indicated.

Conditions of Gas Chromatography

A Beckman GC-2A gas chromatograph with a thermal conductivity detector was used. The output of the TC detector was recorded on a Beckman 10 inch recorder with a 1 mv full scale sensitivity and a recorder speed of 0.5 inches per minute. The GC-2A was run at 1x attenuation, 200 ma, and an oven temperature of 130°C (injection port and detector temperatures are not recorded on this machine). Helium, the carrier gas, was supplied from a cylinder through a two stage pressure regulator. Gas pressure on the machine gauge was maintained at 120 cc/min or 32 lbs pressure during operation. A 6 foot by 1/4 inch stainless steel column packed with Resoflex (Resoflex LAC-1-R-296 Standard Concentration, Burrell Corporation, Fifth Ave., Pittsburgh, Pa.) as the liquid phase was used in the machine.

The syringe for injecting samples into the chromatograph was a Beckman #22405 glass syringe assembly barrel with needle. A 1/16 inch diameter x 3 9/16 inch long brass welding rod with Beckman #97254 teflon tip attached to one end and a knurled knob collar (handle) attached to the other was used for a plunger. To achieve reproducible injections, a plastic block 0.75 inches x 1 inch x 2.25 inches with a 1/8 inch kerf 3/4 inches deep running the length of the block was employed. A 3/16 inch step cut in one end of the block was used to control the amount injected. The syringe was filled by: (a) carefully

drawing an excess of sample into the barrel being certain not to plug the needle with magnesium sulfate; (b) placing the plunger rod into the kerf of the plastic block; and (c) depressing the plunger so that the upper end of the syringe barrel and lower part of the knurled knob were both flush against the plastic block. The last movement expels excess fluid and air from the syringe leaving 14 µl still contained in the barrel (110). The needle was inserted into the syringe inlet of the chromatograph. The knurled knob of the plunger was then slipped from the top of the injection block and pushed to the bottom of the step with one quick motion, thus ensuring the injection of the 14 µl of sample in a single "plug".

Preparation of Samples

From the groups determined by biochemical analysis, organisms were selected arbitrarily for gas chromatographic determination of their metabolic products. Rather than attempt to test all the organisms, a time consuming process, and in order to cover as wide a range of strains of each type as possible, at least one organism of water and one of animal origin was selected from each group. For large groups several organisms of each type were studied. Individual strains not members of any group were also chosen arbitrarily and included to broaden the range of sampling. This was done so values from as diverse a population of organisms as possible might be obtained

in determining the average amount of a metabolic product produced by a type from a particular source for later statistical comparison.

Only with the human strains were gas chromatographic patterns obtained for the entire group. The human group was small (10 organisms) and therefore the entire group was used to provide a valid comparison of their metabolic products to those of strains from wild game.

Preparation of MR-VP media and viable cell cultures for metabolic analysis has already been described. A 5 mm loopful of the bacteria was inoculated into 10 ml of the MR-VP broth under CO_2 using a gas cannula attached to a CO_2 tank as described by the VPI Anaerobe Research Group (110). The system was modified in that no attempt was made to keep the medium O_2 free prior to inoculation. The CO_2 gas was added to reduce the oxygen tension just enough to enhance the fermentation reaction (105) and hopefully the amount of metabolic products formed. The cultures were incubated normally for 5 days at 37°C. This time period was chosen because it corresponds to the incubation time indicated for the methyl red portion of the reaction to be completed (2, 5, 17, 19). It was thought that a more stable amount of end products could be obtained from the various strains if the spent medium was used for testing.

To be certain no products were formed earlier during incubation that would be missed on the fifth day, five (5) cultures were run in quadruplicate and one tube from each tested every day except the fourth day. The fourth day was omitted because the few cultures examined on the fourth day of incubation early in the research failed to show any products different from those found on the fifth day. A parallel set of tubes was prepared and inoculated in the normal manner (without CO_2) to determine if aerobic incubation added to or subtracted from the fermentation reaction.

When it was found a small number of peaks was obtained using MR-VP medium, a new medium, a modified form of Proom and Knight's broth (54, 72, 89), known to yield large numbers of peaks in other gas chromatographic experiments was tried. This medium was composed of three parts: Solution A: KH_2PO_4, 1.5 g; $(NH_4)_2HPO_4$, 7.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $CaCl_2 \cdot 2\ H_2O$, 0.3 g; $MnSO_4 \cdot 4\ H_2O$, 40 mg; $FeSO_4 \cdot 7\ H_2O$, 25 mg; $(NH_4)_2MoO_4$, 2.0 mg; peptone, 10 g; distilled water, 780 ml. The solution was boiled to dissolve the chemicals, filtered and then autoclaved. Solution B: DL-alanine, 380 mg; DL-aspartic acid, 890 mg; L-arginine hydrochloride, 300 mg; L-cystine, 20 mg; L-glutamic acid, 1,400 mg; glycine, 170 mg; L-histidine hydrochloride, 240 mg; DL-isoleucine, 760 mg; L-leucine, 570 mg; L-lysine hydrochloride, 240 mg; DL-methionine, 60 mg; L-proline, 110 mg; DL-serine, 120 mg; DL-

threonine, 60 mg; L-tyrosine, 60 mg; DL-valine, 150 mg; distilled water, 100 ml. Solution C: biotin, 1 µg; folic acid, 2 µg; riboflavin, 100 µg; 500 µg ; each of thiamine (aneurine), nicotinic acid, pyrodoxine and calcium pantothenate. These were made by preparing 10 mg/100 ml stock solution for the first three and 50 mg/100 ml stock solutions of the remainder and then making the necessary dilutions and/or transfers. This solution was then made up to 20 cc with distilled water. Solutions B and C were sterilized by filtering through a millipore filter. To prepare the completed medium, Solutions A, B, and C were combined aseptically with 100 ml of 30% (w/v) autoclaved glucose solution. To enhance the growth of the bacteria, 0.1% peptone (72) was added to Solution A. The medium was tubed in 10 cc amounts into anaerobic culture tubes. The procedures then used with this medium was exactly the same as with the MR-VP broth. Only a small group of ten (10) cultures were tested with this medium. These same organisms were tested in the usual fashion. Again, a small group of four (4) organisms was selected for daily time studies.

After completion of the five day incubation period, all broth cultures were shaken to creat a homogeneous suspension and 6 ml was transferred to a 16 x 125 mm round bottomed test tube. The 6 ml suspension was acidified to pH 2 or below using 0.1 ml (2-3 drops) of 50% aqueous H_2SO_4 (v/v).

For preparation of the volatile fatty acids for analysis 4 ml of the acidified culture was pipetted to a conical centrifuge tube. One ml of ether was added and the tube stoppered. The ether and culture were then mixed by inverting the tube gently 20 times. Next, the mixture was centrifuged briefly to break the ether-culture emulsion. The racks containing the tubes were then placed in the walk-in freezer (-15°C) until the aqueous phase was frozen. The ether layer was then poured into a 12 x 75 mm test tube which contained enough anhydrous $MgSO_4$ to equal about one-half the volume of ether in the tube. The tube containing the extract was then stoppered with 00 rubber stoppers and the mixture allowed to stand ten minutes while the dissolved water in the ether was absorbed by the $MgSO_4$. The ether extract was then left in the refrigerator (4°C) if it was to be run immediately or stored at -60°C if it was not. At temperatures warmer than -60°C it was found the ether volatilized and the sample was lost.

The non-volatile acids were analyzed by formation of their methyl derivatives. Methylation was originally attempted by pipetting one ml of the acidified culture into a conical centrifuge tube and adding 1 ml of Boron Trifluoride (BF_3)-Methanol (Eastman Organic Chemicals) and the mixture allowed to stand overnight. The peaks resulting from both sample specimens and controls from this procedure were unsatisfactory. As a result, the procedure was modified to

include boiling the mixture of acidified sample and BF_3-methanol for five minutes. This was accomplished by pipetting the solutions into round bottom 16 x 125 mm test tubes, stoppering tightly, then placing the tubes in a rack to which a press could be attached firmly over the corks to prevent their blowing out while boiling the tubes in the water bath. The tubes were boiled for five minutes and allowed to stand at room temperature overnight. This method of methylation allowed consistent formation of acceptable peaks. The methylated samples were extracted by pipetting 0.5 ml chloroform into the cooled sample, restoppering, mixing by gentle inversion 20 times, then removing the chloroform layer (bottom layer) with a Pasteur pipette and placing the extract in a 12 x 75 mm tube. Treatment was then the same as for the volatile acids except that the chromatograph column was reconditioned with 14 µl of methanol after every 20 injections of methylated samples.

Controls

Uninoculated media controls were done for each new batch or type of broth. These controls were handled in exactly the same way as the regular samples. These were done to determine background levels of metabolic products that might be present originally in the media.

Quantitative estimations and retention times were determined by preparing multiple standard solutions of known concentrations. Three

sets of these standards were prepared, one each for volatile fatty acids, non-volatile acids, and alcohols. Homologues not expected in the mixed acid reaction were included in order to determine the linearity of the GC-2 chromatographs elutions (69, 93) as well as to facilitate the identification of unexpected chromatographic peaks that might have appeared. Quantitation curves were prepared from the standards to determine the amounts of metabolites produced by the various strains of bacteria. A pyruvate standard was prepared separately from fresh ingredients since the pyruvic acid used in the original standard had deteriorated. Fumaric acid was also prepared fresh to ensure the reliability of this standard since this acid volatilizes on standing. The standards and their contents are listed in Table II.

Control samples were run at the beginning and the end of the chromatographic phase of the project to be certain the sensitivity of the machine had not changed appreciably. The values shown in the standard curve are the result of at least two determinations. Since acetic acid was one of the major components expected in the test samples and is reported to give erratic peaks from sample to sample (93), the value of each standard was determined as the average of quadruplicate samples.

Table II. Standard solutions for gas chromatography.

Compound	Standard Solution Number 1	2	3	4
	amounts in meq/100 ml*			
Volatile Fatty Acids				
Formic	1.0	2.0	4.0	8.0
Acetic	1.0	2.0	4.0	8.0
Propionic	1.0	2.0	3.0	-
Butyric	1.0	2.0	3.0	4.0
Isobutyric	0.5	1.0	2.0	4.0
Valeric	1.0	2.0	3.0	4.0
Isovaleric	1.0	2.0	3.0	-
Non-Volatile Acids				
Pyruvic	1.0	2.0	3.0	4.0
Lactic	1.0	2.0	3.0	6.0
Fumaric	1.0	2.0	4.0	-
Succinic	1.0	2.0	4.0	8.0
Alcohols	amounts in mM/100 ml			
Ethanol	1.7	3.4	6.8	13.6
Propanol	0.5	1.0	2.0	4.0
Isobutanol	0.1	0.1	0.2	0.4
Butanol	0.1	0.2	0.4	0.8

* Contents of the standards are given here in meq for ease in preparation as recommended by the VPI Anaerobe Group. One meq corresponds to 10 μM/ml and will be so referred to in the rest of the paper for consistency during discussion.

The elution of 2,3-butanediol by the methods employed proved impossible. We had hoped this compound would be important in the identification of the --++ group. Two additional procedures recommended for the extraction of this compound were tried. First, the polarity of the medium was increased by the addition of 5% methanol before extraction (L. L. Jackson, Montana State University, personal communication). Second, the solution was acidified with 0.3 M trichloroacetic acid (TCA), centrifuged and injected without following the extraction procedures (G. A. Strobel, Montana State University, personal communication). Neither procedure yielded the appropriate peaks associated with 2,3-butanediol. Injection of a 10% solution of 2,3-butanediol into the machine without extraction or other treatment yielded peaks at a retention time of 9.5 minutes (83). Both this and the 0.3 M TCA procedure produced long, tailing water peaks due to the moisture contained in the sample and would have made the identification of any peaks eluting sooner impossible. With evidence that the column and chromatographic conditions would allow elution of the compound but that our extraction procedures were somehow inadequate, we were forced to exclude the molecule from our fermentation analysis.

Calculations

Standard curves for each of the products formed were prepared from the standard solutions already described. The peaks produced on

the gas chromatograph by each of the compounds in a sample were then identified by their retention times, the area of the peaks determined as outlined below, the areas related to a similar point on the ordinate of the standard curve and the equivalent amount of product formed read from the abscissa of the curve. Area measurements were in square centimeters and quantity of samples in μM/ml. It was thought simpler for our purposes to relate the amount to a similar quantity formed from one of the standards.

Retention times were determined by measuring along the baseline in inches the distance between the injection point and the middle of the peak formed by a compound. Since the chart speed was 0.5 inches/minute, the retention times could easily be converted to minutes.

Curve areas from the chromatographic charts were determined by planimetry (Compensating Polar Planimeter, Keuffel and Esser Co., New York), triangulation, and height x width at half height. The last method was used only in the beginning as a further check on the calculations and for measuring the few peaks obtained not measurable by triangulation. Planimetry was used throughout as a control for the triangulation method. Measurements were taken in quadruplicate by planimetry, averaged and compared with the results from triangulation. Since agreement between the two methods was usually good all values reported in this thesis were obtained by the latter method.

Triangulation is more time consuming, but the precision is reasonable provided the peak shapes allow proper measurement.

Nearly all gas chromatographic peaks have a gaussian shape. The curve is calculated by the triangle formula, area = 1/2 BH, where B = baseline and H = height. The height is measured from the baseline to the intersection of two tangents drawn along the sides of the curve. Limit of sensitivity for considering results as significant was set at a peak size of 10 mm^2 (54, 72, 93). Any area less than this was recorded as a trace (T) and omitted from statistical consideration.

After calculating the areas under the peaks of the compounds formed by the various strains of bacteria tested, the values were arranged according to type and source. Those types from which a number of strains had been tested were compared according to a t (student t) test (98). Comparison of a single observation with the mean of a sample was used for establishing rough relationships between single specimens and a group of similar strains. For example, we wished to compare the single strain of type -+-- of human derivation with the -+-- group from water to determine if any relationship existed. The formulas used for statistical analysis are listed in Table III.

Table III. Method of statistical analysis.

ΣX = Sum of the individual observations.

n = Number of observations.

d.f. = Degrees of freedom (n-1).

$\bar{X}$ = Sample mean ($\Sigma X/n$).

ΣX^2 = Sum of the squares of X.

$(\Sigma X)^2/n$ = Correction term.

Σx = Sum of the squared deviation from the mean

$$X^2 - (\Sigma X)^2/n$$

S^2 = Mean square (variance) = $\frac{x^2}{d.f.}$

s = Standard deviation.

$S^2_{\bar{x}}$ = Variance of the sample mean $(\frac{s^2}{n})$

$S_{\bar{x}}$ = Standard error of the sample mean $(\bar{x})$ $(\sqrt{S^2_{\bar{x}}})$

C.I. = Confidence interval = $\bar{X} \pm t_{.05} S_{\bar{x}}$.

$t_{.05}$ = .05 value in t table at sample d.f.

$t_{.05(df_1+df_2)} = \frac{\bar{X}_1 - \bar{X}_2}{S^2_{\bar{x}_1} + S^2_{\bar{x}_2}}$ = significance level of sample relationships.

Table III. (Cont'd)

Comparison of a single observation with the mean of a sample (99).

$$t_s^* = \frac{\bar{X}_1 - \bar{X}_2}{S_2 \sqrt{\frac{n_2+1}{n_2}}}$$

d.f = $n_2 - 1$

X_1 = Value of the single sample.

$\bar{X}_2$ = Mean of the sample to which X_1 is compared.

S_2 = Standard deviation of the large sample.

n_2 = Number of observations in the large sample.

* It must be stressed that the single test has few, if any, taxonomic implications. It is simply meant to show that we do not have sufficient evidence to reject a hypothesis that a relationship does not exist.

RESULTS

Results of Biochemical Assays: Media and methods were discussed in the preceding section and the results of culture incubation were as described. The reactions yielded by the individual isolates are listed in Appendix A. These results will not be discussed in detail, rather their application to identification of the microorganisms will be emphasized. Pertinent details not included in the appendix, such as pigment production, will be mentioned where applicable.

The EC tests performed on the wild game isolates deserve further comment and the results of these tests are listed in Table IV. This table gives the source of the organism, the number of strains coming from the source, and the number of isolates that are EC positive. The fecal coliform tests for the organisms isolated from water are also listed even though these strains were originally selected on the basis of this reaction and their results, then, are entirely artificial. No control over the animal strains was possible as the EC test had not previously been done on them.

Omitted from Table IV are the ten human strains and the single moose sample (type --++, EC negative). Of the former, 7 of the 10 were EC positive and 1 was of the IMViC type -+-- (EC positive).

Of the 16 deer samples, none is EC positive and only one fits the accepted fecal coliform type, ++-- (*E. coli*). Two are of the -+-- type (*E. coli* var. II). The percentage of positive fecal

Table IV. Reaction of the EC test by type and source.

	Source									
	Water		Elk		Horse		Deer		Bear	
Type	No.	EC+	No.	EC+	No.	EC+	No.	EC+	No.	EC+
-+--	24	6	11	2	2	0	2	0	4	0
++--	96	87	54	45	18	7	1	0	3	3
-+-+	15	2	10	0	3	0	7	0	6	0
--++	8	0	2	0	4	0	4	0	1	0
-+++	6	0	3	0						
-++-	2	0					2	0		
++-+	1	1	8	0	3	0				
+-++	1	0			1	0				
---+	1	0	2	0						

coliform tests for the standard E. coli type from elk was 83.33% while only 2 of the -+-- type yielded aerogenic cultures. Samples procured from equine sources yielded 38.88% positive E. coli. Three of this type were found from bear and all three were positive.

A number of strains that failed to demonstrate a positive fecal coliform test did yield heavy growth in the EC broth at the elevated incubation temperature. A number of these strains, particularly of the -+-- type, were anaerogenic and apparently lacked formic hydrogenlyase. The others, capable of producing gas in carbohydrate media at 37°C, grew at 44.5°C but the enzyme at that temperature remained inactive. Some anaerogenic organisms of the type -+-- also failed to ferment lactose, or fermented it slowly, placing these in the shigella-like class on the basis of the IMViC test, and growth on MacConkey agar (clear, colorless colonies). Lysine decarboxylase tests were positive and the organisms all utilized esculin. Serological tests by the procedures described were also negative. As a result of these biochemical and serological reactions, organisms of type -+--, as a whole, were identified as Escherichia species.

The results from the ++-- group were more typical with only a few strains deviating from the reactions outlined in Edwards and Ewing (22) for Escherichia coli. However, many of the reactions on EMB agar were not typical for these species and a greater than usual

number of the KCN reactions were positive (19, 20). The horse dropping samples 6362H and 6382H are examples of atypical reactions; both were H_2S positive and EC negative. These were not classified as Edwardsiella tarda but left as E. coli because E. tarda ferments only glucose regularly, at times, lactose, and is almost always mucate negative (24, 66). Hence, 6362H and 6382H may be intermediates of the two species. Many of the EC negative strains of this IMViC type were also weak gas producers.

Some biochemical reactions of the E. coli type demonstrated good agreement in matching the organisms from water, elk and other wild game (Table XIV). While in others, there is overlapping of organisms from various sources; for example, the group with strain numbers 2656-6383H show overlapping, with the strains from water, bear, and horse giving similar reactions. At times an individual strain from a particular species will fit in. Isolate number 6384H fits well with the large number of elk and single water samples with which it is shown.

At the beginning of the ++-- group in Table XIV, there are a number of water isolates for which no organisms of similar reactions could be found among mammalian strains. At the end of the data for this same group (Table XIV) there are a number of mammalian strains for which no similar physiological reactions could be found from

water isolates. In the case of the water samples dissimilarities only became apparent when these isolates were matched by numerical taxonomic calculations to others of this type. Even then the group remains fairly homogeneous. Figure 2 shows few S_{SM} values below 70%, the level at which many workers consider subgroups to appear in calculations of this type (85, 100).

Numbers 2438, 6368H, and 6559E gave reactions typical of Proteus morganii. One of these, 2438, was also EC positive.

Type -+-+ demonstrated a wider diversity of organisms that were more difficult to classify. With an exception or two, all the reactions were atypical of any particular genus. The most clear cut distribution was found in the group 2243-6386E. These reactions were typical of the indole negative strain of Citrobacter (10). Sample numbers 2287, 6312D, and 2849-6136E could well be classified within Erwinia except for the fact these were almost entirely non-motile. In the latter group there were also other discrepancies such as failure to ferment glucose, in some cases, few liquified gelatin, and most were non-pigmented.

Three strains, 2913, 2843, and 6311D were oxidase positive. These strains were non-pigmented and according to the King system of classification would be Aeromonas species. These strains were also arginine dihydrolase negative. Organism number 6311D was

phenylalanine deaminase positive and liquified gelatin. Number 6312D appeared to belong with the organisms just described but was oxidase negative and remained so on repeated testing, thus classifying it as possibly an Erwinia (41). Other organisms of this IMViC type were classified as Escherichia species. Still others, we considered unclassifiable, at least on the basis of the reactions performed. A full explanation appears in the discussion.

The isolates appearing to biochemically resemble Salmonella paratyphi A or H_2S negative strains of another serotype, S. enteriditis, serotypes sendai and berta for example (19), were typed with Salmonella anti A, poly A-I, and Vi antisera. These all proved negative.

The -+++ group presented fewer classification problems. None formed gas in EC medium although some did demonstrate heavy growth and few produced a reaction in urea medium. Many of the strains were arginine dihydrolase and ornithine decarboxylase positive. The remainder of the decarboxylase reactions were more typical of E. aerogenes (arginine dihydrolase negative, lysine decarboxylase positive) and E. hafnia (ornithine positive, does not ferment adonitol, inositol or esculin). There did not appear to be any Klebsiella species (lysine positive, arginine, ornithine negative). More difficult to place are those cultures not reacting on any of the

decarboxylase broths.

There were fewer gelatin liquefaction reactions (45%) in these and all groups of this type than are ordinarily expected of the Entero-bacter and related organisms (22, 23). All liquefaction reactions become positive in over 2 and under 12 days although the tubes were held for a full 30 days before discarding.

None of the --++ isolates deaminated phenylalanine or produced H_2S and most grew well in KCN broth. Nearly all produced gas in varying amounts in KIA and carbohydrate media. Only 6194D and 6590E appeared entirely anaerogenic. Five strains produced an acid slant and butt in KIA, the remainder an alkaline slant and acid butt. The type was entirely fermentative. Here, as in some of the reactions produced by other IMViC types, many isolates fermented lactose in purple broth base but apparently not in KIA.

The reactions of type ++-+ seemed to be almost entirely those of Proteus rettgeri. Only one urea negative type appeared. This water isolate was EC positive, had a positive, negative, positive (lysine, arginine, and ornithine respectively) decarboxylase test pattern, did not deaminate phenylaline and was KCN negative. As a result, the organism was classified as an E. coli (22, 24).

Of the nine strains of type -+++ recovered, 6 came from water and 3 from elk. Further subculturing and testing of these organisms in KIA, MacConkey and EMB agar, and the IMViC series were done to determine if these were pure or mixed cultures even though the initial

reisolation procedure had been carefully carried out.

Results of these tests demonstrated 2746, 6517, 2739, 6090, and 6138 to be homogeneous cultures yielding similar reactions. The remainder showed a mixture of colony types on the agar plates. These were picked to KIA and in one case (2838) one tube showed an alkaline slant, acid butt reaction while the other had an acid slant and butt. IMViC reactions were likewise diverse: 2708 and 2712 were -+-+ and --+-; 2521 gave the -+++ reaction; and 6517 and 2838 were -+-- and --++. These organisms were not reclassified.

Several ++++ IMViC types isolated at the beginning of this study were reclassified following reexamination. Reisolation of these cultures showed a mixture of E. coli and Enterobacter types.

Type -++- was found to have the same relationship to deer as -+++ to elk. Two of the organisms were isolated from water and two more from deer. These cultures appeared homogeneous, their reactions best fit those of Klebsiella species (66), although the lysine decarboxylase is negative for strains 2758' and 6592D. Isolate 6592D apparently decarboxylates ornithine slowly and was motile. Since non-motility would seem to be a constant characteristic for Klebsiella, these characters would indicate 6592D may be an Enterobacter instead (66).

The last two small groups, +-++ and ---+, combined contain only five organisms. Isolates 2792 and 6266H of the +-++ type seem best assigned to the E. cloacae while results of 2759 suggests and atypical Citrobacter. Number 6225E of the ---+ group is oxidative and had a positive oxidase reaction. The biochemical reactions indicate this strain may be a Pseudomonas species.

Throughout the biochemical testing program, all cultures were examined closely for possible contamination and gram stains performed in all cases where it appeared contamination might have occurred. All such gram stains examined revealed only gram negative rods. With the indicated exceptions, culture purity was reasonably certain.

Results of Numerical Analysis: Numerical analysis of these organisms using the formula and characters listed revealed some interesting similarities. The results of the calculations are seen in Figure 1 (includes the values for -+--), Figure 2 (++--), Figure 3 (-+-+), and Figure 4 (--++). A list of the group numbers for each of the IMViC types and the laboratory numbers of the isolates composing the groups is found in Appendix B. From Appendix A it can be seen that many more tests were run on each of the isolates than are considered in calculating similarity values. Since common characters may not be considered in the calculations (97), these were omitted. In Figure 2, group number 26 was an isolate for which MacConkey and EMB agar

1	100															
2	80	100														
3	40	50	100													
4	75	75	55	100												
5	45	55	85	50	100											
6	65	65	45	90	40	100										
7	55	75	45	70	40	80	100									
8	40	40	90	55	75	55	45	100								
9	31	42	78	53	74	42	31	78	100							
10	25	35	55	50	40	50	50	55	63	100						
11	55	55	75	60	90	50	40	75	73	50	100					
12	45	55	85	50	90	40	50	75	73	40	80	100				
13	35	55	65	40	70	30	50	55	73	50	60	70	100			
14	50	60	80	65	75	55	55	70	58	60	75	75	55	100		
15	45	45	65	30	70	20	20	55	68	40	60	40	70	55	100	
16	60	60	30	35	25	85	85	40	47	55	35	35	45	40	15	100
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Figure 1 Similarity values of -+-- coliform isolates.

Figure 2. SIMILARITY VALUES OF++-- COLIFORM ISOLATES

| | | |

1	100																	
2	95	100																
3	91	95	100															
4	91	95	100	100														
5	50	55	50	50	100													
6	50	50	50	50	82	100												
7	36	32	36	36	68	77	100											
8	38	29	29	29	71	81	95	100										
9	45	50	55	55	68	68	73	71	100									
10	36	41	45	45	68	68	82	81	82	100								
11	41	45	50	50	64	64	77	76	90	95	100							
12	41	45	50	50	45	45	32	33	62	41	45	100						
13	36	41	45	45	50	68	64	62	82	77	77	68	100					
14	32	36	41	41	55	73	68	62	68	59	66	55	90	100				
15	36	41	45	45	50	68	64	62	82	73	77	68	100	90	100			
16	41	45	50	50	64	55	68	67	77	90	82	55	59	45	59	100		
17	59	64	68	68	45	55	41	33	59	41	45	73	68	66	71	55	100	
18	45	50	55	55	41	50	36	33	59	50	50	95	73	59	73	59	33	100
19	36	41	36	36	59	68	73	81	68	82	77	32	66	59	66	71	33	36	100
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

Figure 3 Similarity values of -+-+ coliform isolates.

1	100													
2	72	100												
3	72	56	100											
4	72	56	78	100										
5	67	53	88	82	100									
6	72	56	89	78	100	100								
7	50	56	33	33	29	33	100							
8	67	50	61	61	65	61	61	100						
9	56	28	61	50	59	61	50	44	100					
10	56	28	61	72	65	61	39	67	67	100				
11	50	33	56	78	71	67	33	50	61	83	100			
12	61	33	67	61	76	78	39	50	72	72	67	100		
13	44	50	44	61	65	61	28	44	56	67	72	72	100	
14	61	44	67	67	71	67	44	83	50	72	56	56	50	100
	1	2	3	4	5	6	7	8	9	10	11	12	13	14

Figure 4 Similarity values of --++ coliform isolates.

reactions were not recorded. The mucate reactions for group 28 of the same fugure was lost. As a result, these reactions were omitted from consideration when comparing these strains with the others. All characters used in determining similarity values were given equal weight in numerical calculations as required by Adansonian principles (97).

These procedures are recommended for use by a computer but for our purposes, these were done manually by listing all matching positive and negative dissimilarity characters for each pair compared and calculating their matching coefficient. As already stated in materials and methods, the values were entered in the diagram with no attempt to group matching types, however, relationships of groups of organisms show up fairly well. As already indicated S_{SM}= 70% is the level at which subgroups appear and strains of a major type (-+--, etc.) showing levels of similarity higher than this may be considered fairly homogenous (4, 85, 100). Fewer characters (eighteen) than expected were used in calculating similarity values for the --++ type. All such isolates utilized salicin and adonitol and were EC negative. Since common characters for a group were omitted from consideration in calculating the values, as explained earlier, these also were omitted. Discussion of these similarities more properly belong in the discussion section and will be considered there.

Gas Chromatography: Prior to analyzing the test organisms with the Beckman GC-2 gas chromatograph, the control solutions were carried through the experimental procedure. The volatile fatty acid solutions eluted as expected and their retention times are listed in Table V. The values shown are the average values determined for the various solutions used. Figure 5 is the graph of the log of the retention time vs. compound carbon number. The elution times for the acids do appear somewhat greater than those obtained for volatile acids from the same type of machine under similar conditions by others (110). Figure 6 is a graph showing the relationship between peak area per milliequivalent injected (the term milliequivalent was retained here for ease in simplifying the relationship) and the number of carbon atoms in the acid molecules. There is linearity between the detector response (increase in peak area) and the increase in the number of carbon atoms. This figure is calculated by dividing the concentration in milliequivalents of the sample injected by the peak area obtained. The graph is plotted as log cm^2/meq vs. carbon number. Formic acid is not included in the last figure and its retention time is slower than would be expected when the linear relationships of the other homologues of the volatile fatty acids are considered.

Table V. Retention times of the known acid standards.

Compound	Retention Time
Formic Acid	7 mins. 45 secs.
Acetic Acid	6 mins. 45 secs.
Propionic Acid	9 mins. 54 secs.
Butyric Acid	14 mins. 23 secs.
Isobutyric Acid	11 mins. 0 secs.
Valeric Acid	22 mins. 33 secs.
Isovaleric Acid	18 mins. 15 secs.
Pyruvic Acid	4 mins. 33 secs.
Lactic Acid	5 mins. 31 secs.
Fumaric Acid	17 mins. 55 secs.
Succinic Acid	21 mins. 50 secs.
Ethyl Alcohol	1 mins. 38 secs.
Propyl Alcohol	2 mins. 14 secs.

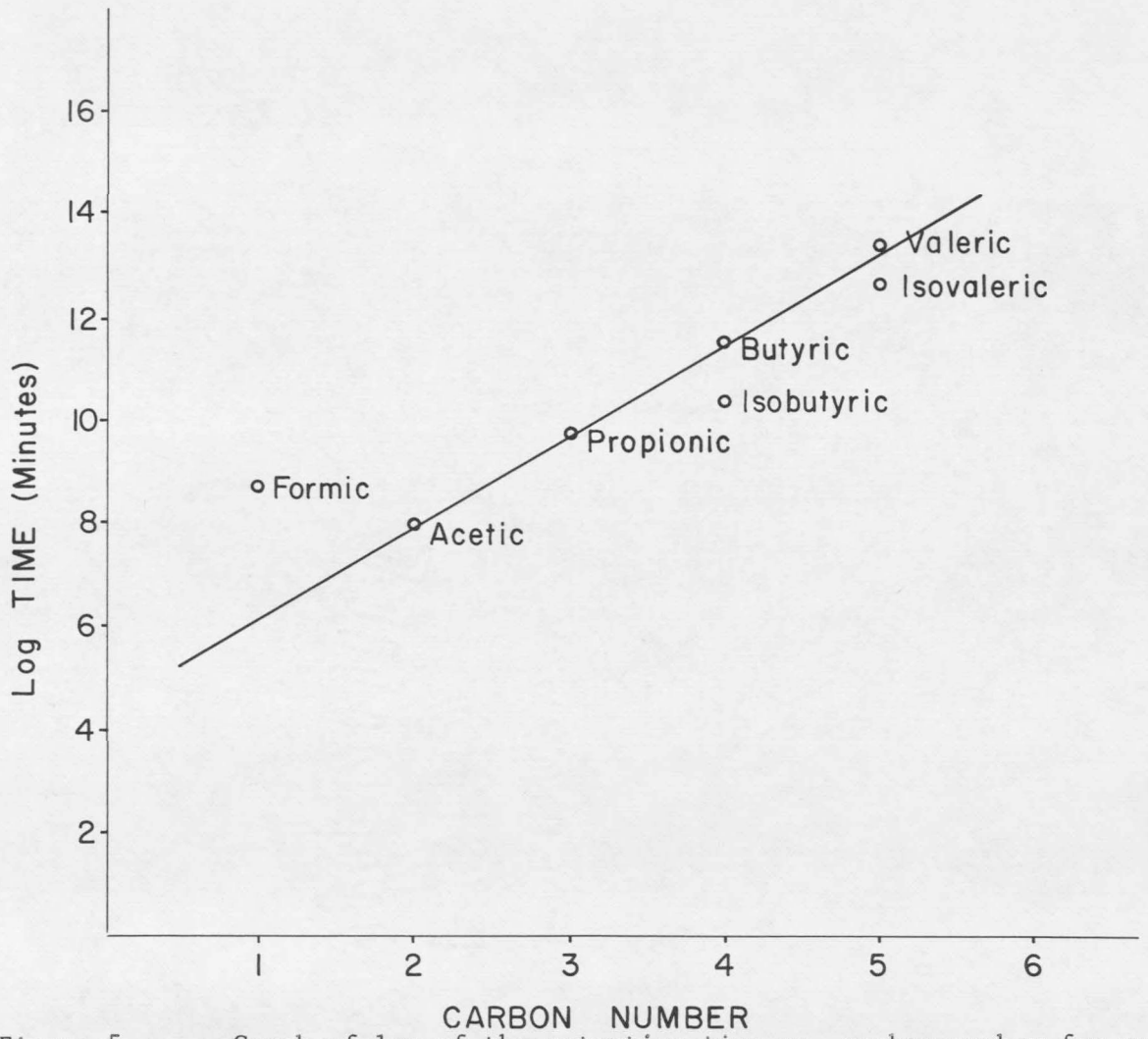


Figure 5. Graph of log of the retention time vs. carbon number for volatile fatty acids.

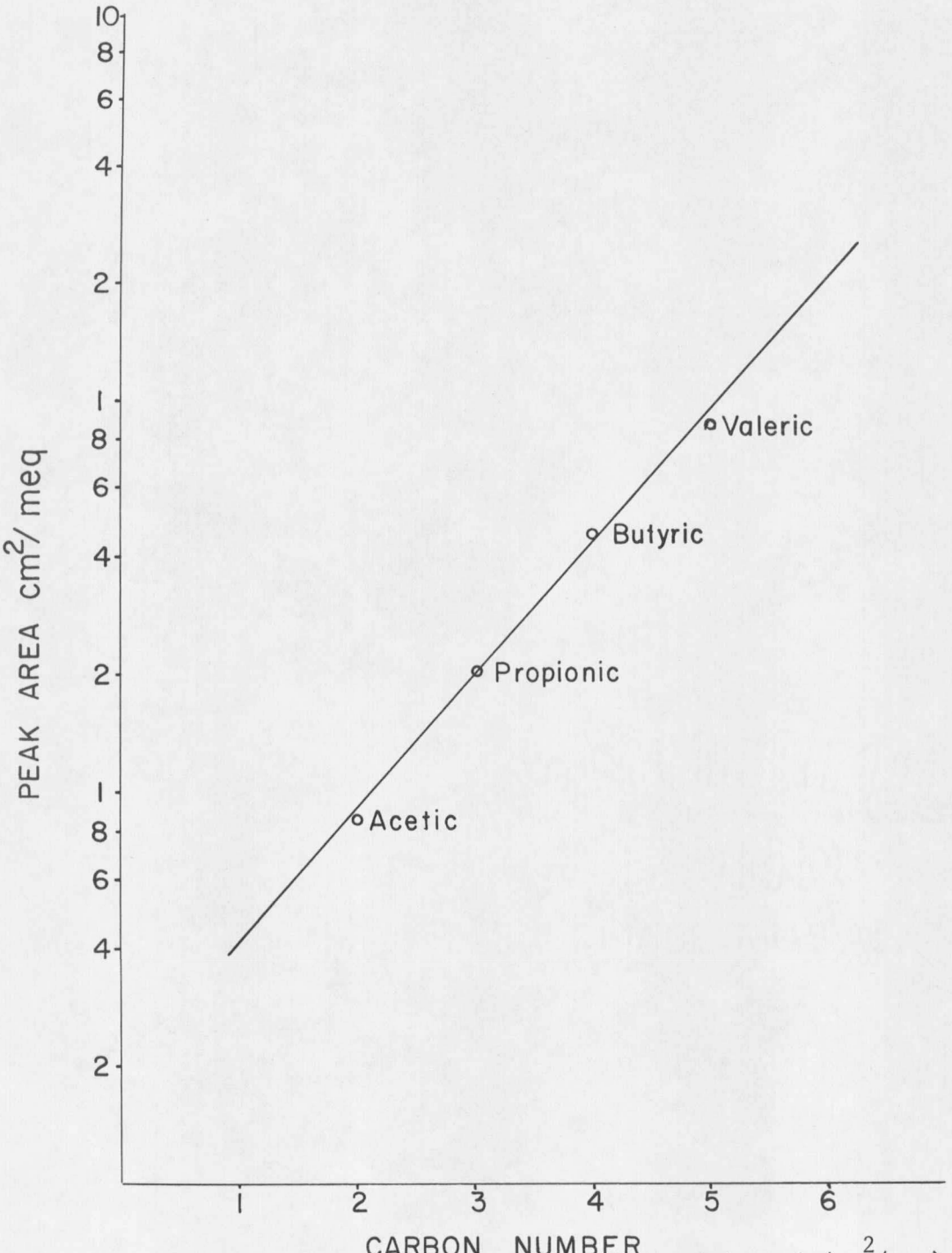


Figure 6. Relation between peak area per meq injected (cm^2/meq) and number of carbon in the acid molecules.

Isobutyric and isovaleric acids are eluted more rapidly than either of the unbranched forms of these acids. These were not included in the cm^2/meq vs. carbon number graph because they presented much the same relationship there.

Figure 7 is a graph showing the linear correlation of peak area to variable concentrations of the volatile fatty acids. Figure 8 shows the slope for acetic acid alone. This was drawn to more easily determine the quantity of this acid in the various cultures. Since acetic acid gives more erratic elution results, the values shown are the average of six determinations. It will be noted that the lines, while linear, do not project to the origin (zero concentration) as they should.

The determination of non-volatile acids was not as simple as for the volatile fatty acids. The peaks obtained from the methylation procedure used were reproducible. The pyruvic acid standard, prepared fresh, gave peaks of good linearity. Figure 9 shows linear correlation of peak areas to variable concentrations of non-volatile acids. Figure 10 was prepared so that lactic acid and pyruvic acid quantities formed by the fermentation activity of the coliform isolates could be determined from the peak areas. Again orientation of the lines toward the origin of the graphs is not good.

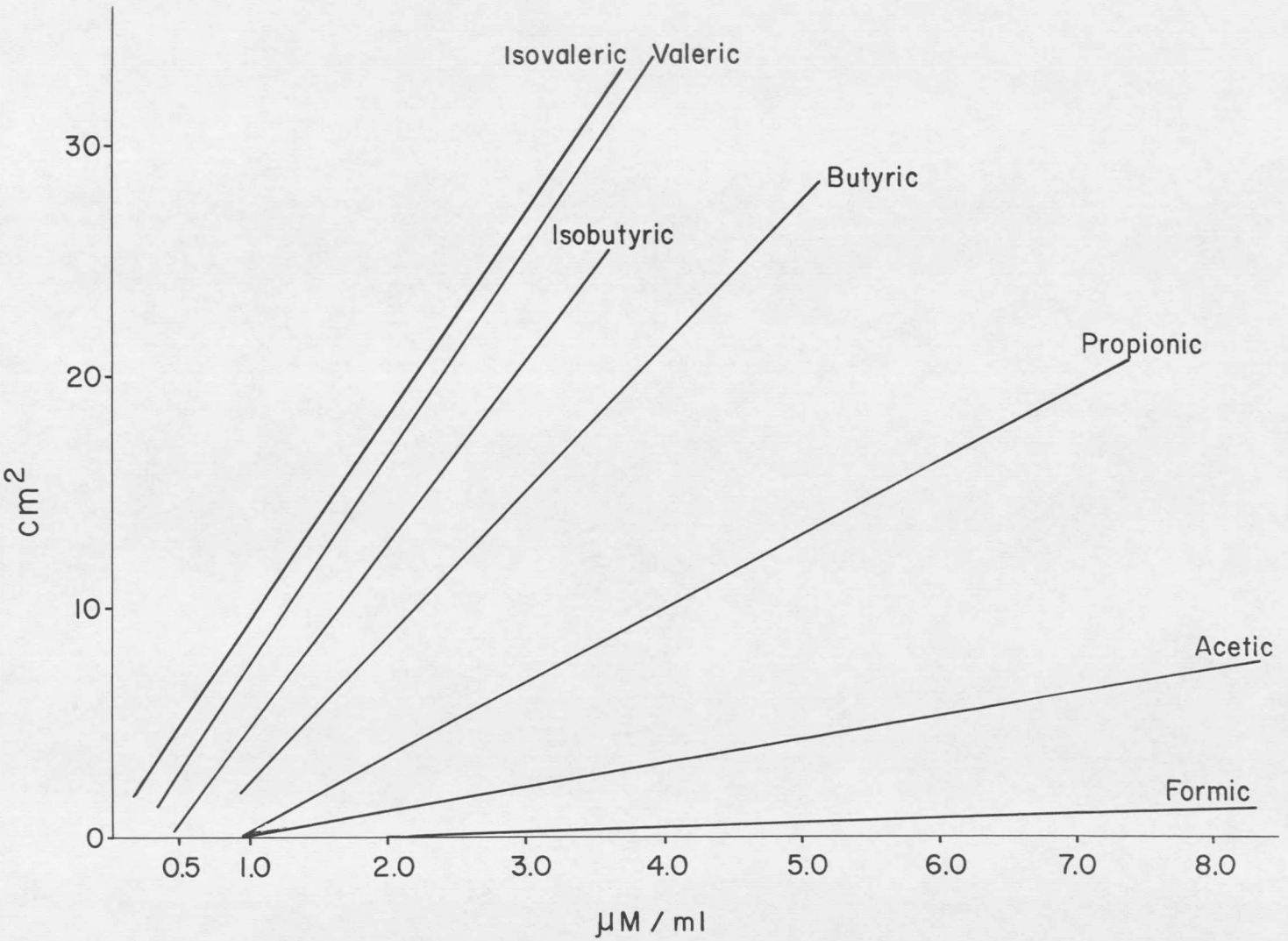


Figure 7. Linear correlation of peak area to variable concentration of volatile fatty acids.

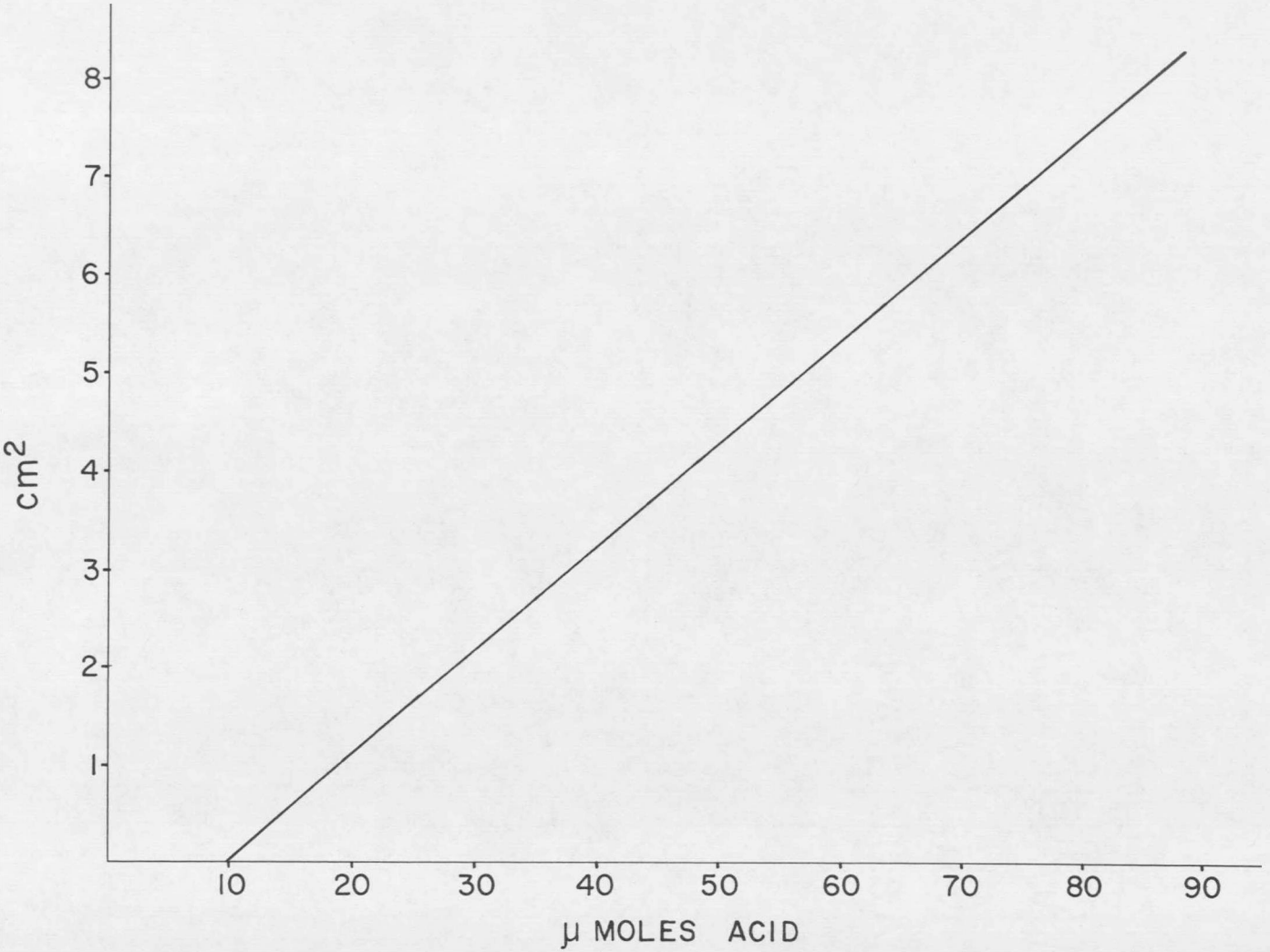


Figure 8. Linear correlation of peak area to acetic acid concentration.

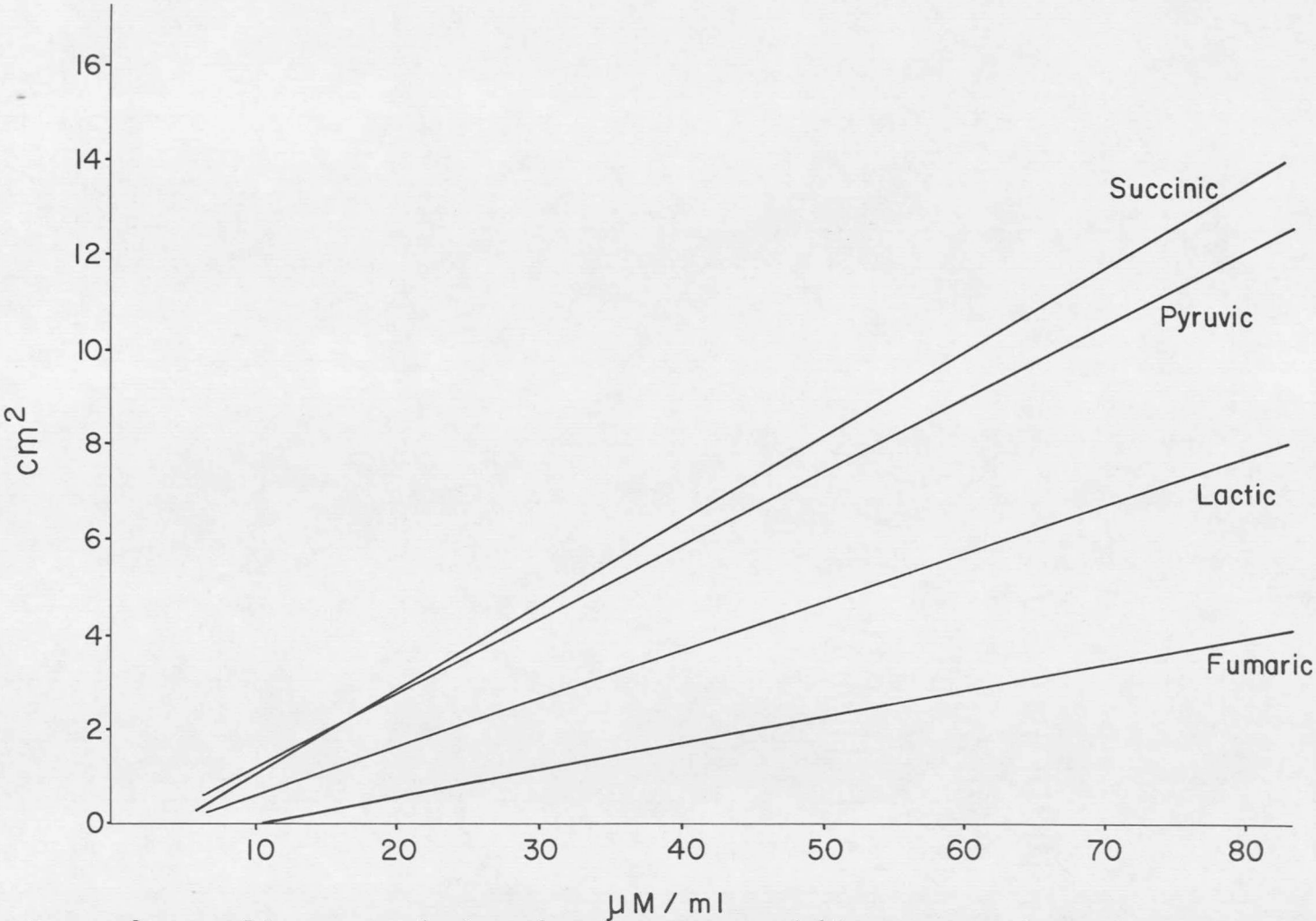


Figure 9. Linear correlation of peak area to variable concentrations on non-volatile acid standards.

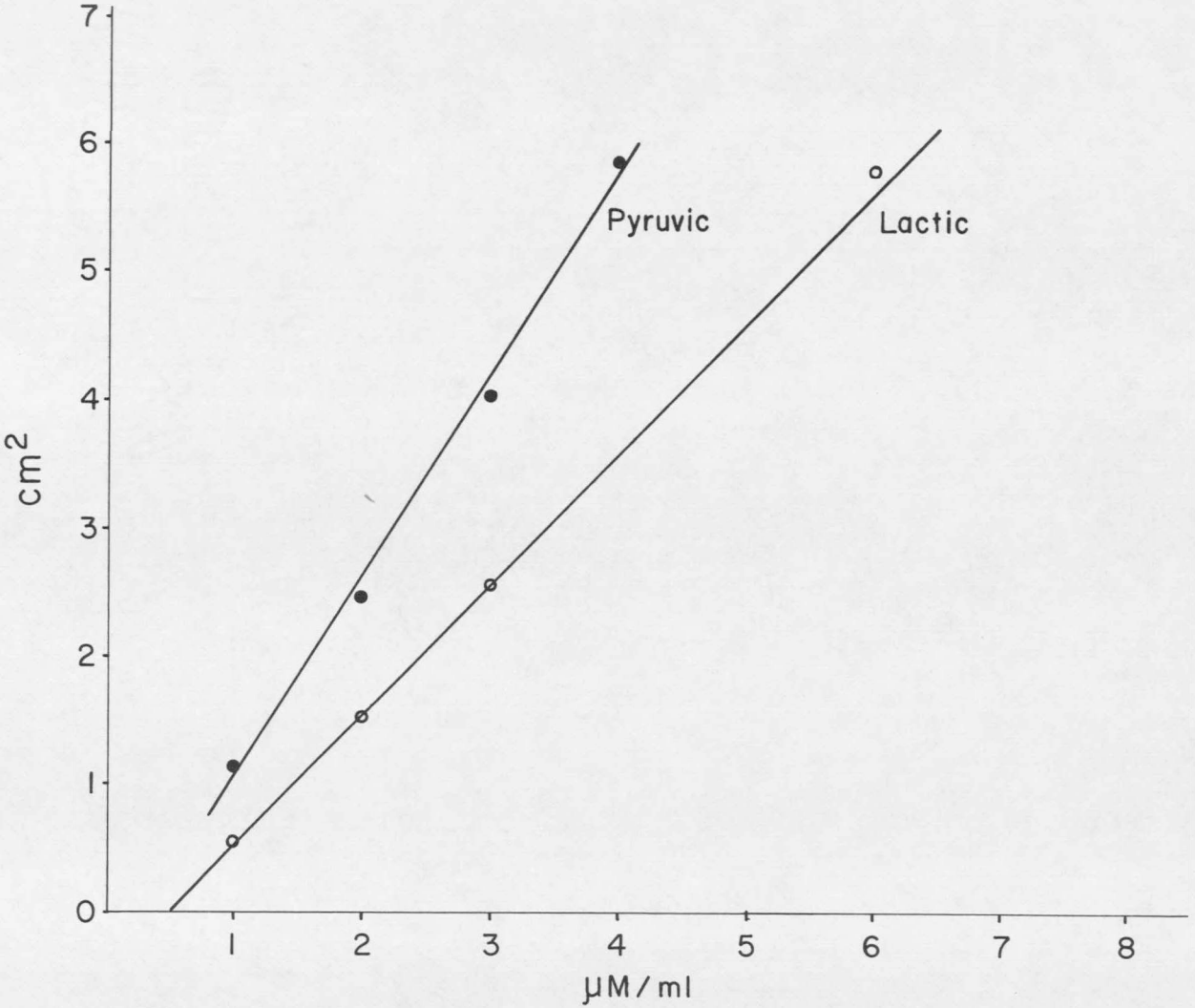


Figure 10. Linear relationship of peak area to lactic and pyruvic acid concentrations.

Extracts of uninoculated broth used for growing the isolates were also prepared and injected into the chromatograph. There were no peaks from the media, either for the volatile acid precedure or the non-volatile acid one.

Alcohol determinations also presented difficulty. Peaks equivalent only to those of ethanol and n-propanol were obtained; peaks for the remainder were not obtained even after allowance for a prolonged retention time (83). With this procedure, elution of alcohols appeared to be inadequate (110). The graph of the variable concentration for those alcohols recovered is seen in Figure 11. No alcohols were found to elute from the fermentation products of the coliform isolates. This was surprising in view of the finding by Cecchini (14) and the fact that in the Voges-Proskauer reaction, ethanol, in theory, is present in increased concentration (103).

The injection procedure was modified as experience was gained so that samples were injected at 11-12 minute intervals instead of at the end of 23 minutes by which time valeric or succinic acids, if present, would have been eluted. The ether and chloroform solvents were found to elute immediately after injection in a single peak without effecting the retention time of compounds still going through the column. Thus, an eleven minute schedule allowed elution of the solvent, the acetic, pyruvic, and lactic acid peaks, if

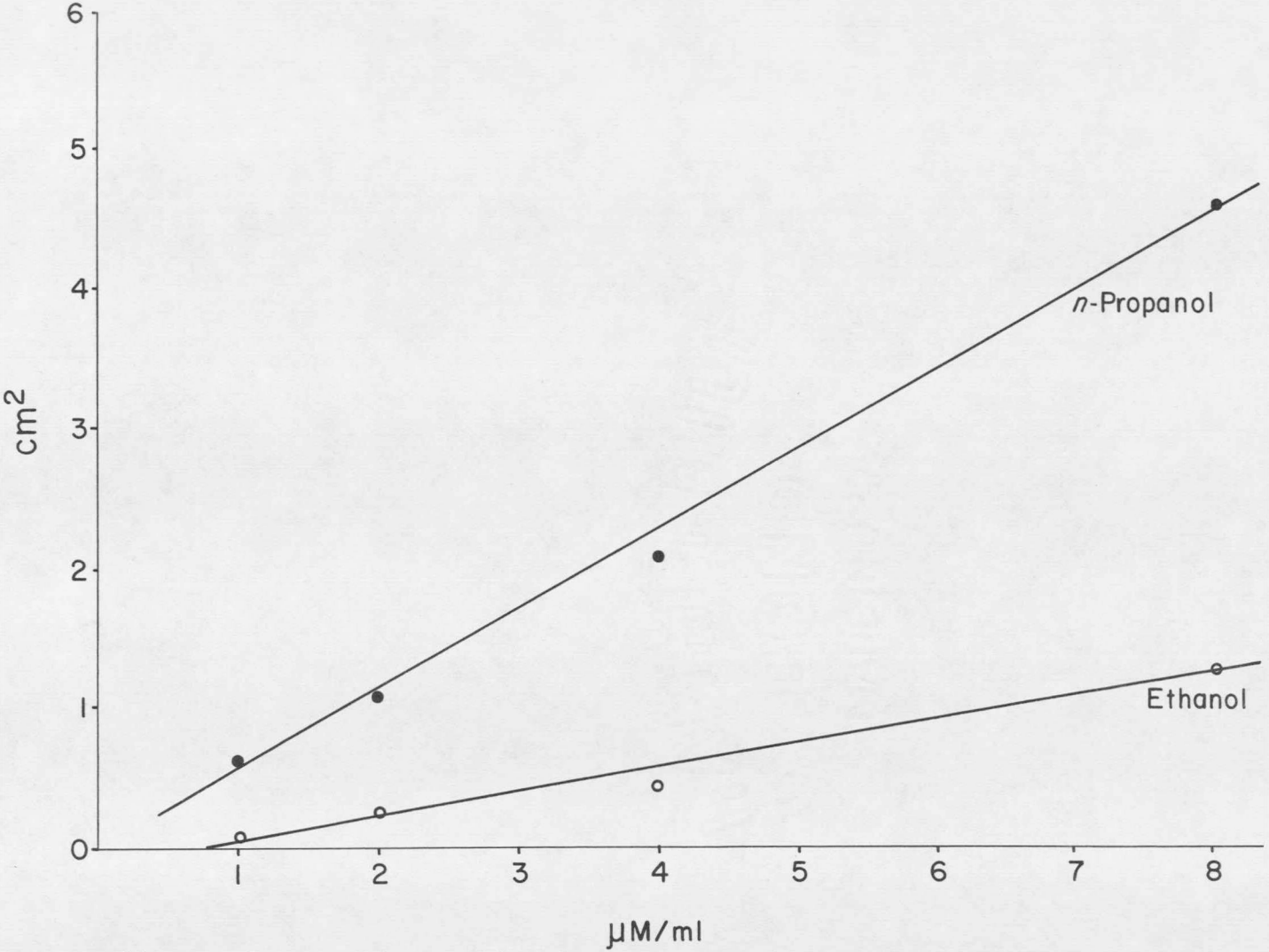


Figure 11. Linear relation of peak area to variable concentrations of alcohol standards.

present, and still leave the peaks of compounds with longer retention times easily identifiable. The tailing water peak immediately following the solvent peak was never a problem with the procedures used.

Leakage in the machine was prevented by changing the silicone rubber diaphragm, through which the samples were injected, after each 50 injections. The diaphragm was changed sooner if the recorder stylus seemed to become erratic. When tanks of helium were changed all tubing leading to the machine developed a leak and this required resealing of that part of the system. These were the only complications that ever developed with the GC-2 chromatograph.

The syringes used for drawing the samples were kept in the freezing compartment of the refrigerator between injections. If they were allowed to warm, it was impossible to draw the ether and chloroform into the barrel.

Injection of methanol into the machine after each 20 chloroform extract injections to clear the column, appeared to keep the peaks sharp and within the determined elution time.

Ether extracts, in particular, proved difficult to keep. It was found that plugging the 12 x 75 mm test tubes in which the extracts were kept with number 00 rubber stoppers and freezing at -60°C prevented volatilization of the samples before they could be run. The

samples were removed from the freezer 6 at a time when being tested in the chromatograph and allowed to warm in the refrigerator.

During the chromatography of the actual isolates, it was found that even those organisms yielding good peaks, uniformly yielded only acetic and lactic acids. At times a trace of pyruvic, formic or succinic acids would appear. Most organisms produced a flat "plateau" on the recorder chart at the position of pyruvic acid, but no actual peak resulted. A few organisms such as 6197E produced no fermentation products of any kind. This matched its biochemical reactions. It grew only on citrate and formed acid with no gas in glucose and lactose.

To determine reproducibility of peaks formed by the gas chromatograph, random samples were selected for retesting, some from the same extract, and others were retested from freshly inoculated cultures. It has already been indicated that a fair degree of reproducibility was obtained upon reinjection of the known standards. Reproducibility of the samples from the isolates showed much less uniformity. Samples reinjected from the same extract were fairly reproducible, those from fresh cultures of the same isolate demonstrated a wider range of variability, from a trace to more than that found in the original culture. For example, 6519E yielded 10.00 uM of acetic acid initially, when repeated it yielded only a trace. Number 2458 had acetic acid values of 22.00 and 17.00 uM; 2613, 19.00 and 19.00 uM; and 2521 gave acetic

acid values of 13.00, 14.00, and 15,000 uM on triplicate tests. Similar values are seen in the tables for lactic acid.

The time studies, in which multiple cultures of the same organisms were run at daily intervals, revealed no new peaks. The same variation in peak sizes as those from the spent media (incubated for 5 days) was found. Those tests handled normally (no CO_2 added) gave similar results.

The Proom and Knight medium prepared as described and inoculated as were the regular cultures also showed a lack of other peaks. In some cases, succinic acid became observable but the size of the acetic and lactic acid peaks were no larger than those yielded by the regular method.

The peak areas obtained for each of the acids produced by the organisms are shown in Tables VI through XI. The organisms are listed according to type (-+--, ++--, etc.) with the isolates separated according to source to make statistical evaluation simpler. Separate tables are provided for the -+--, ++--, and -+-+ types since these contained the largest number of strains tested. The remaining types are composed primarily of single strains from a given source so these were all placed under miscellaneous in Table IX. The results from the Proom and Knight medium are listed in Table X. The organisms selected for this procedure were chosen entirely at random and no attempt was

Table VI. G.C. analysis of acid products of type -+---.

Strain Number	Acetic Acid		Lactic Acid		Formic Acid	Pyruvic Acid	Succinic Acid
	Area*	Quant**	Area	Quant	Quant	Quant	Quant
Water							
2317	.25	12.0	.65	11.0			
2740	.48	14.0	1.00	14.00			
2750	.20	12.0	.77	12.0	T		
2943	.08	T	.60	11.0			
2791	.43	13.0	.67	12.0			
2435	.18	11.0	.70	12.0			
2518	.32	12.0	.90	14.0			
2361	.28	12.0	.67	12.0			
2430	.90	18.0	1.30	18.0			
2433	.07	T	.66	11.0			
2449	.06	T	1.15	16.0			
2449	-	T	.69	12.0			
Horse							
6364H	1.00	19.0	1.12	16.0			11.50
6364H	1.43	22.0	1.16	16.0			-
Human							
9-14035A	1.08	19.0	1.40	19.0		T	
Elk							
6230E	-	T	1.83	23.0			
6178E	.20	11.0	.70	12.0			
6156E	1.05	19.0	1.00	15.0			
6176E	.33	12.0	.68	12.0			
6034E	.20	11.0	.48	10.0			
6519E	.14	11.0	.68	11.0			
6519E	.06	T	.75	12.5			
6392E	.55	14.0	.50	10.0			
6520E	.38	13.0	.23	7.0			
Bear							
6306B	.10	10.0	.63	11.0			
6305B	1.43	22.0	1.54	20.0			

* Number represents centimeters squared.
** Number represents micromoles.

Table VII. G.C. analysis of acid products of type ++--.

Strain Number	Acetic Acid		Lactic Acid		Formic Acid	Pyruvic Acid	Succinic Acid
	Area	Quant	Area	Quant	Quant	Quant	Qunat
Water							
2250	.90	18.0	1.12	16.0			
2458	1.23	22.0	.80	13.0			
2458	.86	17.0	.65	12.0			
2309	.44	14.0	1.24	17.0			
2310	1.55	23.0	1.41	19.0			
2311	1.48	23.0	1.42	19.0			
2286	.63	15.0	.95	14.0			
2295	.75	16.0	.99	15.0			
2648	1.36	22.0	1.29	18.0			
2554	.45	14.0	.45	9.0			
2561	.40	13.0	1.04	15.0			
2320	1.36	22.0	1.13	16.0			
2303	.42	13.0	1.15	16.0			
2304	1.62	25.0	1.23	17.0			
2655	.49	14.0		14.0			
2655	.52	14.0		14.0			
2655	.44	13.5		12.0			
2654	1.08	19.0	1.15	16.0			
2549	.38	13.0	1.12	16.0			
2664	1.22	21.0	.98	14.0			
2569	.36	13.0	1.28	18.0			
2613	1.08	19.0	1.00	18.0			
2613	1.06	19.0	1.00	14.0			
2432	1.20	21.0	1.48	20.0			
2432			1.10	16.0			
2652	1.40	23.0	1.45	19.0			
2459	1.45	23.0	1.10	16.0			
2459	1.63	25.0	.76	12.0			
2933	.30	12.0	1.28	18.0			
2517	1.08	19.0	1.00	15.0			
2548	.38	13.0	1.05	15.0			
Elk							
6390E	.82	17.0	.88	14.0			
6506E	.78	17.0	1.18	16.0			
6389E	.92	18.0	1.65	21.0			

Table VII. (Cont'd)

Strain Number	Acetic Acid		Lactic Acid		Formic Acid	Pyruvic Acid	Succinic Acid
	Area	Quant	Area	Quant	Quant	Quant	Quant
6389E	1.22	21.0	1.10	16.0			
6252E	.73	16.0	.95	13.0			
6511E	.75	16.0	1.12	16.0			
6256E	.05	T	1.03	15.0			
6162E	1.02	19.0	1.43	19.0			
6326E	1.22	21.0	1.58	22.0			
6297E	1.38	22.0	1.38	18.0			
6292E	1.05	19.0	1.60	21.0			
6505E	.75	16.0	.86	14.0			
6328E	.57	15.0	1.55	20.0			
6509E	1.60	25.0	.54	10.0			
6168E	1.22	21.0	1.00	15.0			
6164E	1.45	23.0	1.48	19.0			
6164E	1.43	23.0	1.30	17.0			
6159E	1.10	19.0	.95	14.0			
6159E	1.08	19.0	1.28	18.0			
6550E	.30	12.0	.42	9.0		6.0	
Horse							
6370H	1.65	25.0	1.20	17.0			
6372H	.64	15.0	1.15	16.0			
6362H	2.52	33.0	.75	12.0			
6365H	1.61	24.0	1.92	24.0			
6253H	1.50	23.0	1.43	19.0			
6253H			1.05	15.0			
6384H	.83	17.0	.40	9.0			
Bear							
6307B	1.73	25.0	1.40	19.0			
6290B	1.45	23.0	1.28	18.0			
Deer							
6313D	.49	14.0	.85	14.0			
6313D	.24	11.0	.68	12.0			
Human							
4-17225A	1.40	23.0	1.61	20.0		T	T

Table VII. (Cont'd)

Strain Number	Acetic Acid		Lactic Acid		Formic Acid	Pyruvic Acid	Succinic Acid
	Area	Quant	Area	Quant	Quant	Quant	Quant
5-2266A	1.19	20.0	1.20	17.0			
2-2535A	1.38	22.0	1.65	21.0			
1-14607A	1.58	24.0	1.32	18.0			
3-2229B	1.87	27.0	1.70	22.0			
8-1516	1.98	28.0	1.62	21.0		T	
6-16974A	1.37	22.0	1.40	19.0			
7-1940A	1.66	24.0	1.19	17.0			T
10-15431B	.94	18.0	1.47	19.0			T

Table VIII. G.C. analysis of acid products of type -+-+.

Strain Number	Acetic Acid		Lactic Acid		Formic Acid	Pyruvic Acid	Succinic Acid
	Area	Quant	Area	Quant	Quant	Quant	Quant
Water							
2287	.75	16.0	.40	9.0			
2429	.35	12.0	1.00	15.0			
2835	.38	13.0	1.00	15.0			
2843	.40	13.0	1.10	16.0			
2849	.66	15.0	1.10	16.0			
2948	.50	14.0	1.28	18.0			
2072	.14	11.0	1.05	15.0			
Elk							
6385E	1.20	21.0	1.43	19.0			
6131E	.20	11.0	.70	12.0			
6174E	1.66	25.0	.90	14.0			
6195E	.15	11.0	.80	13.0			
6136E	.08	T	.90	14.0			
6030E	.66	15.0	.70	12.0			
Deer							
6311D	.42	1310	.85	13.0			
6314D	.56	15.0	.90	14.0			
6310D	.38	13.0	.47	10.0			
6195D		-		-			
Bear							
6308B	.81	16.0	.43	9.0			
Horse							
6366H	.80	16.0	1.40	18.0			

Table IX. G.C. analysis of acid products of miscellaneous coliforms.

Strain Number	Acetic Acid		Lactic Acid		Formic Acid	Pyruvic Acid	Succinic Acid
	Area	Quant	Area	Quant	Quant	Quant	Quant
--++							
Water							
2318	.71	16.0	.67	12.0			
Deer							
6552D	.23	11.0	.18	6.0			
6593D	.70	16.0	.33	7.0			
Elk							
6590E	-	-	.13	6.0			
Bear							
6594B	.29	12.0	.24	7.0			
Moose							
6196M	.78	17.0	.67	12.0			
Horse							
6275H	.40	13.0	.05	T			
-+++							
Water							
2521	.11	13.0	.17	6.0			
2708	.56	15.0	.60	11.0			
2708	.42		1.20				
Elk							
6138E	.10	T	.68	12.0			
6090E	.34	13.0	.59	11.0			
6517E	.22	11.0	.57	11.0			
++-+							
Water							
2315	.88	18.0	1.33	18.0			
Horse							
6267H	.48	14.0	.23	7.0			

Table IX. (Cont'd)

Strain Number	Acetic Acid		Lactic Acid		Formic Acid	Pyruvic Acid	Succinic Acid
	Area	Quant	Area	Quant	Quant	Quant	Quant
Elk 6583E	.52	14.0	.54	10.0			
+-++ Water 2792	.84	18.0	.74	12.0			
Horse 6266H	.90	19.0	.68	12.0			
-++- Water 2836	.46	14.0	.60	11.0	1.0		

Table X. Results from Proom and Knight medium.

Strain Number	Acetic Acid		Lactic Acid		Formic Acid	Pyruvic Acid	Succinic Acid
	Area	Quant	Area	Quant	Quant	Quant	Quant
6594B	.39	13.0	1.56	20.0			12.4
6266H	1.07	18.0	.99	15.0			14.8
6197E	-	-	-	-			-
6297E	1.10	19.0	2.01	24.0			13.0
2320	.90	18.0	2.40	28.0			9.0
2521	T	T	.84	10.0			T
2708	.26	12.0	1.15	17.0			-
6196H	-	-	.84	13.0			8.1
2554	.50	12.0	1.84	23.0			6.0
6195D	.90	18.0	1.00	15.0			T

made to isolate them by type although this may still be determined by the number or number suffix.

Statistical Analysis: Statistical comparisons were carried out entirely by the formulae given in materials and methods. Such comparisons were thought preferable since the paucity of peaks made "fingerprinting" impossible. For each type, the water organisms and those from animals comprising groups of two or more were totalled, the means calculated, and the confidence intervals estimated. The results of these calculations are found in Tables XI and XII. It is apparent that for 3 or more samples, calculations with narrower standard deviations of sample means and confidence intervals may generally be obtained. For tests involving two samples, unless a narrow range of values was obtained, confidence intervals were not measurable. An example of this is the interval calculated for bear of the -+-- type. Since multiple tests per sample were not run the values obtained for all the samples of an individual source were calculated instead; for instance, the 11 water samples and 7 elk isolates of type -+-- were averaged and the statistical parameters calculated for each of these. The duplicate tests run on certain samples when determining the reproducibility of results by the gas chromatographic procedure were not considered in statistical analysis. As a rule, the first sample run was the one used in the analysis. Compounds showing only a trace from certain of the isolates

Table XI. Results of statistical analysis of acetic acid from G.C. data.

Type and Source	n	ΣX^2	$(\Sigma X)^2/n$	Σx^2	s^2	$s^2_{\bar{X}}$	$\bar{X}$	$\bar{X} \pm t_{.05} s_{\bar{X}}$
-+--								
Water	8	1386.00	1352.00	34.00	4.86	.61	13.00	13.00 ± 1.85
Elk	7	1233.00	1183.00	50.00	8.33	1.19	13.00	13.00 ± 2.92
Bear	2	584.00	512.00	72.00	72.00	36.00	16.00	16.00 ± 76.20
++--								
Water	25	854.00	8100.00	440.00	18.33	.73	18.00	18.00 ± 1.77
Elk	6	5642.00	5476.00	166.00	11.07	.69	18.50	18.50 ± 1.77
Horse	6	3333.00	3128.00	205.00	41.00	6.83	22.83	22.83 ± 5.18
Human	9	4886.00	4807.00	79.00	9.88	1.23	23.11	23.11 ± 3.42
Bear	2	1154.00	1152.00	2.00	2.00	1.00	24.00	24.00 ± 12.70
-+-+								
Water	7	1280.00	1262.29	17.71	3.00	.43	13.43	13.43 ± 1.59
Elk	5	1533.00	1377.80	155.20	38.75	7.55	16.60	16.60 ± 7.61
Deer	3	563.00	560.33	2.67	1.34	.45	13.66	13.66 ± 2.88
--++								
Deer	2	377.00	364.50	12.50	12.50	6.25	13.50	13.50 ± 31.60
-+++								
Water	2	394.00	392.00	2.00	2.00	1.00	14.00	14.00 ± 12.70
Elk	2	290.00	288.00	2.00	2.00	1.00	12.00	12.00 ± 12.70

Table XI. (Cont'd)

Type and Source	n	ΣX^2	$(\Sigma X)^2/n$	Σx^2	s^2	$s^2_{\bar{X}}$	$\bar{X}$	$\bar{X} \pm t_{.05}s_{\bar{X}}$
Water	46	12700.00	12186.96	513.04	11.00	.25	16.14	16.14 ± 1.00
Elk	32	8894.00	8064.50	829.50	26.76	.84	15.88	15.88 ± 1.88
Horse	11	4676.00	4320.36	355.64	35.56	3.23	19.55	19.55 ± 4.01
Human	10	5247.00	5152.90	94.10	10.46	1.05	22.76	22.76 ± 2.37
Bear	6	2138.00	1944.00	194.00	38.80	6.47	18.00	18.00 ± 6.53
Deer	6	1136.00	1120.67	15.33	3.06	.51	13.67	13.67 ± 1.84

Table XII. Results of statistical analysis of lactic acid from G.C. data.

Type and Source	n	ΣX^2	$(\Sigma X)^2/n$	Σx^2	s^2	$s^2_{\bar{X}}$	$\bar{X}$	$\bar{X} \pm t_{.05} s_{\bar{X}}$
--+--								
Water	11	1911.00	1859.00	52.00	5.20	.47	13.00	13.00 ± 1.54
Elk	8	1412.00	1250.00	162.00	23.14	2.89	12.50	12.50 ± 4.02
Bear	2	521.00	481.00	40.00	40.00	20.00	15.50	15.50 ± 56.79
++--								
Water	25	6503.00	6368.00	135.00	5.63	.23	15.96	15.96 ± 0.97
Elk	17	4712.00	4481.94	231.06	14.44	.85	16.24	16.24 ± 1.95
Horse	6	1707.00	1568.00	139.00	27.80	4.63	16.17	16.17 ± 7.33
Human	9	3390.00	3364.00	26.00	3.25	.76	19.33	19.33 ± 1.38
Bear	2	685.00	684.50	.50	.50	.25	18.50	18.50 ± 6.35
--+-+								
Water	7	1592.00	1545.00	47.00	7.83	1.12	14.86	14.86 ± 2.59
Elk	6	1210.00	1176.00	34.00	6.80	1.13	14.00	14.00 ± 2.73
Deer	3	465.00	456.33	8.67	4.34	1.45	12.33	12.33 ± 5.16
--++								
Deer	2	85.00	84.50	.50	.50	.25	6.50	6.50 ± 6.35
-+++								
Water	2	157.00	144.50	12.50	12.50	6.25	8.50	8.50 ± 31.70
Elk	3	386.00	385.33	.67	.34	.11	11.33	11.33 ± 1.43

Table XII. (Cont'd)

Type and Source	n	ΣX^2	$(\Sigma X)^2/n$	Σx^2	s^2	$s^2_{\bar{X}}$	$\bar{X}$	$\bar{X} \pm t_{.05} s_{\bar{X}}$
Water	49	10896.00	10618.50	277.50	5.78	.12	14.61	14.61 ± 0.67
Elk	36	7856.00	7225.00	631.00	18.03	.50	14.17	14.17 ± 1.43
Horse	10	2480.00	2250.00	230.00	25.55	2.55	15.00	15.00 ± 3.59
Human	10	3751.00	3724.90	27.10	3.10	.31	19.30	19.30 ± 0.70
Bear	6	1336.00	1176.00	160.00	32.00	5.33	14.00	14.00 ± 5.94
Deer	6	739.00	682.67	56.33	11.24	1.87	10.67	10.67 ± 3.52

were omitted from consideration since trace amounts were not thought measurable (54, 72, 93). This explains why n is smaller for some of the groups in the statistical tables than for the same groups of isolates in the acid production tables.

To establish a general comparison between metabolic products for coliforms from two sources, the acetic or lactic acid values of all the strains from a particular source were added and these compared to all the products from another source. Thus, all the coliforms from elk were compared to all those from water, horses, etc.

Table XIII contains the t value relationships of these organisms. Next to the computed t value for each figure is shown, in parentheses, the approximate position the value occupies in the t table (100) and consequently the probability of the two samples being related. Samples whose t values lie on or nearly on a table value or lying outside the table figures for .001 or .90 relationship have only the single table value shown.

The relationships are more clearly shown by the bar diagrams in Figures 12 through 15. In these figures the data for the fecal and total coliform acid products have been extracted and compared using the P value relationships of the various sources. Emphasis is placed on relating the water and human isolates to those from other sources. Figure 12 demonstrates the probability that organisms defined as fecal

Table XIII. $T_{.05(df_1 + df_2)}$ values of G.C. data by type and source (100).

df_1 Type and Source	df_2 Type and Source	$T_{.05(df_1 + df_2)}$ Acetic Acid	Lactic Acid
-+--	-+--		
Water	Elk	0.00 (P = >.90)	.27 (.90≥P >.50)
	Bear	.49 (.90 >P >.50)	.52 (.90 >P >.50)
++--	++--		
Water	Elk	.42 (.90 >P >.50)	.27 (.90 > P >.50)
	Horse	1.76 (P = .10)	.10 (P = >.90)
	Human	3.65 (P = .001)	4.41 (P = <.001)
	Bear	.60 (.90 >P >.50)	3.68 (P = .001)
Elk	Horse	1.58 (.20 >P >.10)	.03 (P = >.90)
	Human	3.32 (.01 > P >.001)	2.81 (P = .01)
	Bear	4.23 (P = < .001)	2.68 (.02 > P > .01)
Horse	Human	.10 (P = >.90)	1.36 (P = .20)
	Bear	4.23 (P = < .001)	2.68 (.02 > P > .01)
Human	Bear	.60 (.90 >P > .50)	.60 (.90 >P >.50)
-+-+			
Water	Elk	1.12 (.40 > P > .20)	.57 (.90> P >.50)
	Deer	.24 (.90 > P >.50)	1.58 (.20 > P >.10)
Elk	Deer	1.04 (.40> P > .20)	1.04 (.40 > P > .20)
≶Water	≶Elk	.25 (.90 >P > .50)	.56 (.90 >P >.50)
	≶Human	5.71 (P = < .001)	7.15 (P = < .001)
	≶Horse	1.83 (.10 > P >.05)	.24 (.90 >P >.50)
	≶Bear	.72 (.50 > P > .40)	.26 (.90 >P >.50)
	≶Deer	2.83 (.01 > P >.001)	2.79 (P = .01)
≶Elk	≶Human	5.01 (P = < .001)	5.70 (P = < .001)
	≶Horse	1.83 (.10 > P > .05)	.47 (.90> P >.50)
	≶Deer	1.90 (.10 >P >.05)	2.27 (.05 > P >.02)
	≶Bear	.78 (.90> P >.50)	.07 (P = >.90)

Table XIII. (Cont'd)

df_1 Type and Source	df_2 Type and Source	$T_{.05(df_1 + df_2)}$ Acetic Acid	$T_{.05(df_1 + df_2)}$ Lactic Acid
≶Horse	≶Human	1.55 (.20>P>.10)	2.54 (P = .02)
	≶Bear	.50 (.90>P>.50)	.36 (.90>P>.50)
	≶Deer	3.05 (.01>P>.001)	2.06 (.10>P>.05)
≶Human	≶Bear	1.74 (.20>P>.10)	2.24 (.05>P>.02)
	≶Deer	7.27 (P = < .001)	5.83 (P = < .001)
≶Bear	≶Deer	1.64 (.20>P>.10)	1.24 (.40>P>.20)

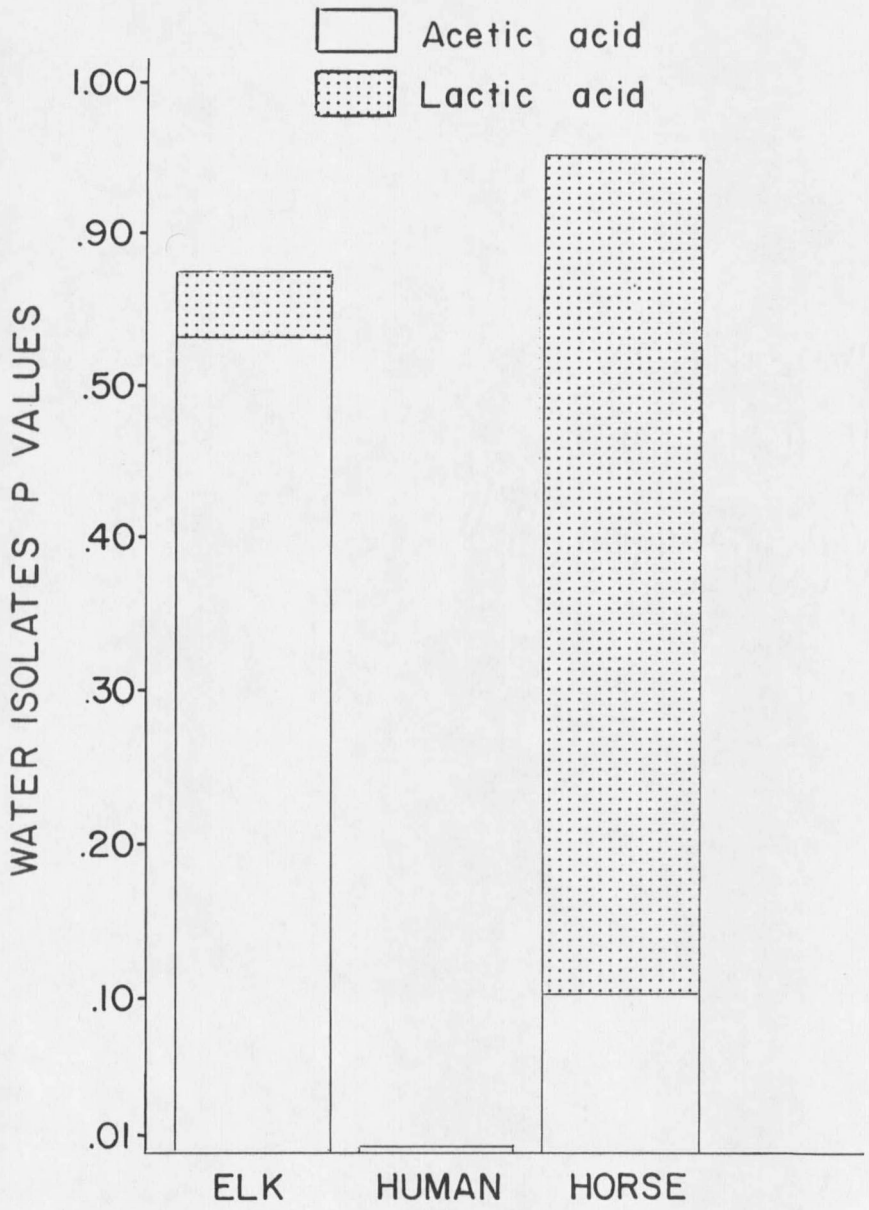


Figure 12. Probability of relationship of water fecal coliform isolates to those from mammalian sources.

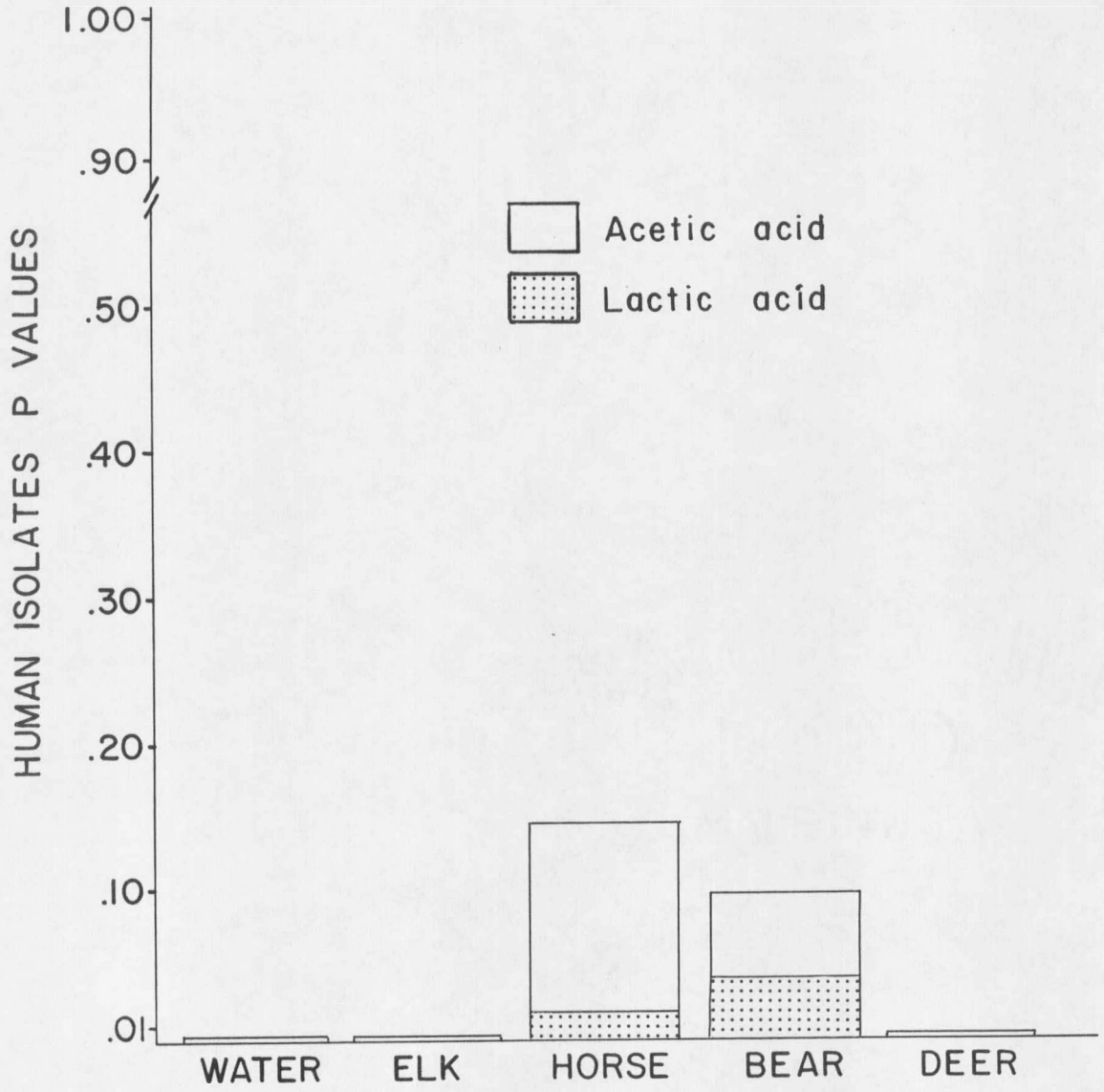


Figure 13. Probability of relationship of human fecal coliform isolate to those from other sources.

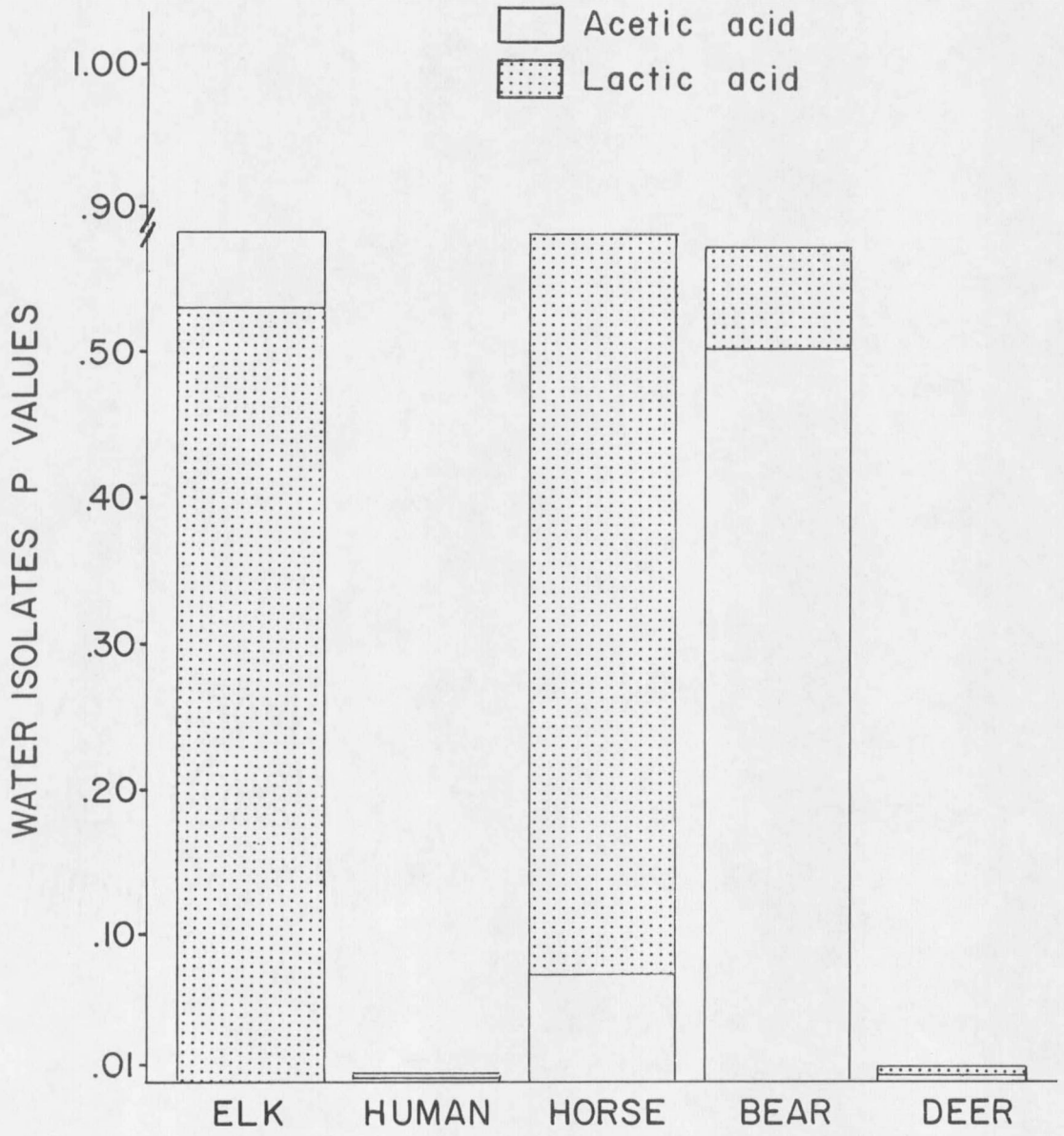


Figure 14. Probability of relationship of all coliform isolates from water to those from mammalian sources.

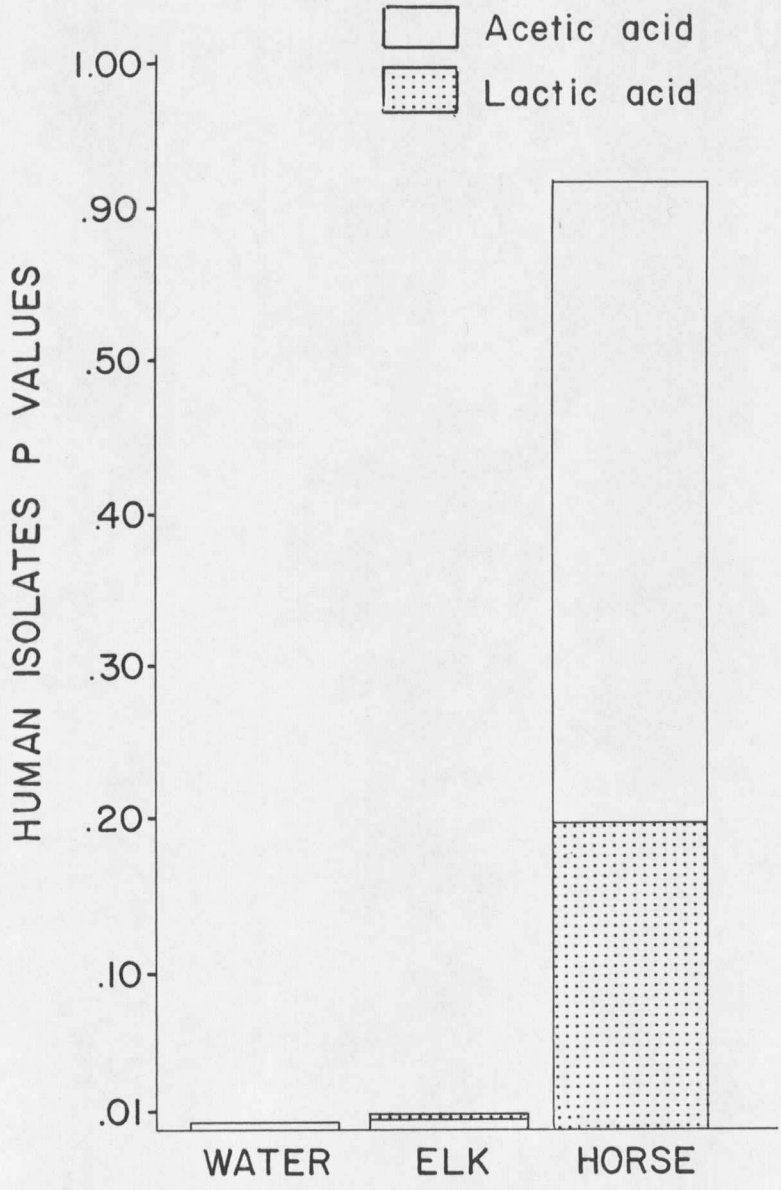


Figure 15. Probability of relationship of all human coliform isolates to those from other sources.

coliforms come from elk even though the lactic acid P value from horse isolates is closer to that of water. The data for bear and deer fecal coliforms are not included in the diagram due to the small number of isolates from these sources. Figure 13 shows the probability of acid products from human E. coli being related to those of any other source. Figures 14 and 15 indicate the relationship between the acid products of all coliforms isolated from water (Fig. 14) and humans (Fig. 15) to those from other sources. Again bear and deer isolates have been omitted from Figure 15 because the majority of organisms isolated from these sources were not of the fecal type and no other types from human sources were used.

In the cases where only a single strain was isolated from a particular source the values are given in the text of the discussion when they are considered applicable. Calculation of these was carried out by comparing the single value with the mean of a group as shown in materials and methods. In view of the variation in the amount of products obtained, this text was used sparingly and will be discussed later. We were forced to include the strains from type --++ in this single category because many of the cultures designated for use in this study expired. This group designated as a soil microorganism by many and one expected to be more resistant to adversity proved the type from which strains were most readily lost during storage.

DISCUSSION

Several workers (25, 52, 65) have found enteric pathogens in the water and sediments of streams in remote areas as well as in the intestinal tracts of small mammals living in watersheds drained by these streams. Although no pathogens were found in the current study, there remains a need for the identification of sources of fecal contamination. The lack of standard fecal coliforms in species such as deer suggests that the indicator organisms signifying the presence of fecal contamination may also need redefining. The currently accepted indicator organism of water potability is *E. coli*, IMViC type ++--, which usually gives a positive EC test. This study has indicated that the presence of this organism may not necessarily serve as an indicator of pollution where some species of wild game are concerned as a variety of IMViC types were actually isolated from these sources. The significance of some of these will require further investigation.

All such organisms were immediately classified by the Elizabeth King System which relies on the O/F and oxidase reactions. The methods adapted from Edwards and Ewing (22) and Martin (66) for biochemical identification of the microorganisms then permitted the identification of most of the isolates to the generic level. The exceptions to this were mentioned in the results section and will be commented upon further here. It must be emphasized that the methods adopted for use

in this study were derived from medical texts for organisms coming from an environment generally different from the one studied here.

The fecal origin of many of the organisms may also be questioned. They were collected from animal droppings found lying on the ground or from the water of the streams fed by the surface runoff from the area. Six and seven isolates from bear and deer, respectively, of the comparatively few obtained from these sources are of the type -+-+. This is the type Geldreich (37) describes as constituting 48.1% of all coliforms isolated from undisturbed soils. This is also the type commonly associated with Salmonella organisms. Both H_2S negative and positive organisms of this type were found with the latter identified as Citrobacter sp. These were isolated from a variety of sources including water, bear, horses, and elk. They are also described as being found in a variety of other sources in nature (10), hence, it is difficult to attach any significance to them. The rest of the strains of this type are difficult to classify. Washington et al. (113) have described H_2S negative variants of Citrobacter but none of the organisms from this study seem to fit their description well enough for inclusion in this genus. The reactions of several (2072, 6314D, and 6315D) would seem to best fit E. coli. At least 1.0% of this species are indole negative; 0.3% may be citrate positive, and 1.0% are KCN positive (22). The positive ornithine reactions of these strains

exclude any of the Klebsiella species and positive esculin and negative gelatin reactions would generally exclude E. cloacae (66, 67, 68, 91, 116). According to the King system of classification, -+-+ strains 2843 and 6311D should be Aeromonas species since they are fermentative, oxidase positive and were unpigmented. Aeromonas, however, is arginine dihydrolase positive and is negative for both lysine and ornithine decarboxylase reactions (43, 56, 82). Number 6312D is more likely to be an Erwinia, even though non motile (41). Providencia would also fit well here since phenylalanine deaminase is positive, but Providencia is also generally indole positive, KCN positive, gelatin negative, and ferments either adonitol or inositol, while this organism does not. It has been reported that for these reactions Providencia yields erratic results (107) and any or all could be negative.

Strains 2898 and 2951 demonstrated yellow pinkish pigmentation. The pigmentation plus the biochemical reactions of the pair indicate they are Erwinia. An exception would be the slowly positive ornithine decarboxylase reaction of strain 2898. The presence of possible Erwinia sp. confirms the soil or water origin of at least some of the -+-+ type.

The wide diversity of possible identifications, the apparent importance of the type as a member of the fecal flora of both deer and bear, as well as displaying physiologic properties of Salmonella and

Arizona make final classification of all organisms comprising the -+-+ type essential.

Some of the reactions for the types from which few organisms had been isolated were clearcut. All but one of the ++-+ have already been described as P. rettgeri. The water strain 2315 of this type is likely a citrate-positive E. coli (22). The +-++ types are considered to be Enterobacter; 22.0% of Enterobacter strains have been found to be indole positive (116). Klebsiella is also frequently indole-positive, but again these cultures are ornithine decarboxylase-positive and motile.

The -+++ and -++- strains have already been discussed in the results section. For those -+++ strains found not to be mixed cultures, as IMViC pattern such as this has been reported in the literature for E. cloacae (68). The decarboxylase-negative and motility-positive strains could also be Erwinia since many of these are methyl red-positive as well as producing acetylmethylcarbonol. Both these types, being specific for individual species as they seem to be (elk for -+++ and deer for -++-), might have interesting implications if they were not so low in numbers in nature and, therefore, relatively difficult to isolate.

Certain isolates of the --++ strains, such as 2377 and 2380, being motile and negative on all decarboxylase reactions, would include

Erwinia species as another general type to be found in this group along with Enterobacter species (10). E. cloacae is described (10) as being found in human and animal species as well as sewage, soil, and water. E. aerogenes has been reported from similar origins but more predominately from grains, plants, and soil (10, 96). That all isolates of the --++ type were uniformly EC negative, even though many were of fecal origin in this case, makes them unlikely indicators of fecal contamination in the wild. That none of these organisms seemed to be Klebsiella supports the findings of Dufour and Cabelli (22) who showed that only this genera from the --++ type can be expected to be EC positive.

Organisms of the types -+-+ and --++ were found to be the coliform strains constituting the majority of organisms found in the fecal flora of deer and with the exceptions of the -++-, two of type -+--, and a lone ++--. These findings were supported by unpublished data (author unknown). This report stated that coliform organisms were sparse in deer fecal samples and that these were not of the standard type. A research project involving the fecal flora of deer would do much to clear up the confusion. This is essential due to the already described potential of deer as carriers of pathogens (3, 20, 92).

The -+-- were found to be 20.4% positive in EC testing. Although this figure may be somewhat inaccurate due to the biased fashion in

which water sample strains were chosen, it still matches the 21.8% level of positives reported for this type (34). Two of 11 elk isolates were positive and while the sample is small it still approximates the expected recovery from fecal material.

The lack of Shigella among the -+-- type supports the belief that Shigella organisms are derived primarily from humans. As indicators of fecal contamination, strains of the -+-- show some promise since the data show these to be consistently found in all species. The type has not been reported to include organisms other than the Shigella and E. coli var. II. Some Erwinia are reported to be only methyl red-positive (41) and characteristics of organisms such as 2430 and 2431 of this type could well fit the description. However, Erwinia are usually motile, have never been reported EC positive, liquify gelatin in higher percentage than shown here, decarboxylates no amino acids and are generally pigmented. All these characters would seem to exclude the genus from the current group. The low level of positive EC tests need not be considered too seriously since the data show a majority of the strains to be anaerogenic and, therefore, lacking in formic hydrogenlyase. This, of course, does take away the advantage of having a simple test available for their identification.

Coliform type ++-- was the largest group examined and is, of course, the type representing E. coli, the common fecal coliform.

The type, as a whole, presented the greatest homogeneity, with the few exceptions already described, and ease of identification. Apparent lack of this type in deer, however, would seem to prevent its being considered as a universal pollution indicator. All reactions were as expected for E. coli (10, 22, 24, 96), but still diverse enough to make determination of similarity coefficients for these organisms interesting. Some of the isolates demonstrated an atypical growth pattern on EMB agar (85), a character that was considered in the calculation of similarity values.

The level of positive EC tests for the elk fecal coliforms (83.5%) approaches those reported for human, cattle, and other animals (34, 70). For some reason, those isolated from horse droppings were only 38.8% positive. Admittedly, the 18 E. coli isolates examined is a small sampling but it would be interesting to know if this is a constant characteristic. Other reports discussing this aspect could not be found. Most of the results available for domestic animals are for cattle. The single E. coli isolate recovered from deer was EC negative. The 3 E. coli isolated from bear droppings were EC positive, but these were only 21.6% of all the organisms recovered from bear. None of the other organisms found in wild game droppings responded to the test, with the exception of the -+-- strains from elk. This evidence certainly supports our original position that new fecal

pollution indicators need to be found.

The results of numerical analysis of the taxonomic groups demonstrated that organisms from the same species, such as E. coli, but isolated from diverse sources are, for the most part, physiologically indistinct. Only in isolated cases, such as the single E. coli (++--) isolated from deer (group 43, Fig. 2) were low levels of relationships shown to all the other (++--) isolates. In only one case did this strain show fairly good relationship to another organism, if one accepts the 70% level as the significant level of relationship at which sub-groups might be separated from groups (88, 94, 97). Group number 25 in Figure 2 also appears unrelated to the other ++-- groups and has a similarity value to group 43 of $S_{25.43} = 75\%$. No other strains in this figure show such individuality in their relationships. Group 24 of Figure 2 representing an elk strain has a similarity value of $S_{24.45} = 80\%$ to group 45, the three EC positive E. coli from bear. It also has a correlation of $S_{24.31} = 80\%$ and $S_{24.53} = 75\%$ to groups containing elk and human strains respectively. The other relationships over 70% shown by the group is for organisms isolated from water.

Groups 44-47 of Figure 2 are from water, bear, horse, and elk and all these organisms group nicely with each other and taxonomic groups 1-8, 16-22, and 26-33 at levels of similarity, in most cases,

of between 70-90%, with many in the 80-85% range. As a final illustration of overlapping relationships within the ++-- type of organisms, group number 16 of Figure 2 is derived from water. It has matching coefficients with other groups of $S_{16.32}$ = 90%, $S_{16.17}$ = 90%, $S_{16.18}$ = 85%, $S_{16.48}$ = 45%, $S_{16.49}$ = 40%, $S_{16.52}$ = 90%, and $S_{16.53}$ = 90%. Appendix B shows that group 32 is from elk, groups 17, 18, 48, and 49 are from horses and 52 and 53 from humans. Similar results can be found throughout the diagram.

A like situation exists in Figures 1, 3, and 4 for the rest of the IMViC types. A random sampling of the overlap shows, in Figure 1, matching coefficients of $S_{5.11}$ = 90% and $S_{5.12}$ = 90%; number 5 is a group containing physiologically similar water and elk isolates. Groups 11 and 12 come from elk and deer respectively. Also in Figure 1, one finds the values of $S_{6.16}$ = 85% and $S_{7.16}$ = 85%. These values represent the relationships of horse and elk strains, respectively, from groups 6 and 7 to group 16, a human isolate.

In Figure 3 (type -+-+), is largest number of uniform relationships are groups 1-4 at levels of 91-100%. These are the Citrobacter and come from water, bear, horses, and elk. $S_{3.4}$ = 100% are from horse and elk strains respectively. Elsewhere in the diagram, a good grouping is found for groups 7-11 with the highest levels at $S_{9.11}$ = 90% and $S_{10.11}$ = 95%. All these were from a single physiological

group as determined by the biochemical tests and groups 9, 10, and 11 are deer, bear, and elk respectively. Group numbers 7 and 8, the water strains from the same physiological grouping, had a value of $S_{7.8} = 95\%$, but were related to the mammalian isolates at lower levels of between 70 and 80%. Such data show that coliforms of diverse origins can show a similarity in physiologic types. An occassional similarity value does stand out for individual pairings, for example, $S_{12.18} = 95\%$ are for two deer isolates. These organisms show similar matching coefficients with no other groups. The highest similarity values next to the one shown is $S_{13.18} = 73\%$ (sample 13 is from water) and $S_{15.18} = 73\%$ (sample 15 is from an elk).

The type with the lowest general levels of relationships is seen in Figure 4. Here, for the --++ strains, the highest levels of relationships are at 88 and 89%, all for water isolates. It is interesting that $S_{8.14} = 83\%$, the next highest similarity value in the diagram is for a deer isolate (no. 8) paired with the one from moose (no. 14). With regard to $S_{1.2} = 72\%$, group 1 is from water and 2 is from deer. Group 2 has matching coefficients of 28-56% with all other organisms of the type. From the emerging pattern, it would appear that deer isolates are the only ones we can identify from the physiologic groupings. However, $S_{9.12} = 72\%$ are strains

from deer and bear respectively.

With exception of the higher relationships pointed out in Figures 1, 3, and 4 and if again one accepts the 70% level at which subgroups may be identified (88, 94) a large number of organisms exist in these types that have little relationship to each other, even in strains coming from similar sources. Organisms of -+-+ type have little resemblence to common Enterobacteriaceae. This type is known to contain some of the most medically significant organisms, as well as the most innocuous of as yet apparently unidentified soil micro-organisms. For the most part, numerical analysis of all the types merely pointed up the lack of randomness with which the physiological types had been grouped. High similarity values between groups serve merely to emphasize the physiological similarities of organisms of diverse origins. Only with an occasional deer isolate could mammalian coliforms be identified with water derived strains using numerical taxonomy.

It would seem that good methods for distinguishing between sources of the strains cannot be found using physiological reactions and numerical analysis. We must then turn to other tools. Serological identification was proven quite effective in the past but here could prove difficult. If the antigens of these organisms are as diverse as physiologic studies indicate, preparing specific antisera against

them would be a time consuming task indeed. As an alternative we considered the use of gas chromatography; if applied to selected isolates of all the available types, this method may yield quantitative data that would allow differentiation of these microorganisms.

To adequately characterize a bacterial species, a number of strains from a variety of sources should be examined (83). For this reason it was thought best to obtain GC data on the metabolic products of as many of the isolates as possible, rather than obtaining data on only a few of the strains. In view of the paucity of identifying bacterial signatures formed by the organisms tested, gas chromatographic comparison of the isolates were based on the relative amounts of acetic and lactic acids formed by each. Only in the case of the strains from human sources could trace amounts of other acids be found. The special medium of Proom and Knight did not seem to yield enough additional information in spite of its complexity to warrant its routine use.

With glucose as carbon source, the composition of the medium in which the cells were grown has been found to be of little importance in obtaining distinct bacterial signatures. O'Brien (83) reported the same results whether he used brain heart infusion broth, trypticase soy broth or peptone yeast extract broth.

Such a paucity of peaks, as we obtained, should have been expected. The mixed acid fermentation reaction yields only formic, acetic, lactic, and succinic acids, and 2,3-butanediol (acetoin may be included as an intermediate). The method of analysis we used was one disigned to give good separation of the volatile and non-volatile acids and alcohols found in the anaerobic system. It apparently does not elute other compounds. The large range of compounds obtained by other workers were achieved by use of liquid phases such as Carbowax 4000 in the column and most often flame ionization detectors, in one case a tritium foil detector was used (83). The acid-ether extraction technique was also used in some cases. The Proom and Knight medium has likewise been shown to yield numerous peaks for fingerprinting bacteria when electron capture detectors and flame ionization detectors are used. Again the columns were packed with Carbowax 4000. Used with electron capture detectors Carbowax 4000 columns proved particularly sensitive in the presence of acetoin. While these columns proved sensitive to alcohols and such compounds as acetoin, acetic, lactic, and formic acids were not recovered (71, 72).

Loss of 2,3-butanediol was considered surprising. The compound has been recovered by others using acid-ether extraction procedures on their media (72, 77). That the Resoflex column used would pass the compound, at least in large amounts, was shown by the two peaks

at approximately 10 minutes retention time after the injection of a 10% solution of 2,3-butanediol. Other workers using Porapak Q (117) have been able to recover the compound from unextracted culture broths. Porapak Q is a porous polymer composed of ethylvinylbenzene cross linked with divinylbenzene (69). The polymer also detects the other common acids found in our cultures, acetic, lactic, pyruvic and formic acids (117). Since it elutes water in a narrow peak, the moisture peak doesn't interfere with the peaks eluting soon after the solvent peaks. This then effectively eliminates the need for pretreatment procedures if these interfere with the elution of the compound.

Loss of acetoin and 2,3-butanediol may best be explained by the fact that many columns of the diphasic (liquid and solid support phases) type adsorb hydroxylated compounds (55). The newer monophasic columns prepared from uncoated porous polyaromatic beads such as the Porapak Q described above allow hydroxylated compounds to pass more readily (8, 55).

It has been mentioned that the standard curves prepared against known quantities of volatile acids in Figure 7 do not project to zero as they should to signify zero concentration. Formic acid could not be detected in the machine prior to the injection of standards containing 40 μM/ml. Acetic acid was not detectable in amounts much

under 10 μM/ml. Other evidence that the method used was of low sensitivity is the failure to detect acetic acid in extracts of uninoculated culture broth. These peaks have been commonly reported by other workers (77, 93, 117). A combination of the two factors, adsorption and procedure insensitivity, could have prevented detection of the amounts of acetoin and/or 2,3-butanediol formed in the culture media.

Also surprising was the amounts of acetic and lactic acids detected in cultures of the --++ type including Enterobacter. The respective amounts of these acids reported in standard reference works to be produced by Enterobacter is so low that it should probably be considered to be nondetectable with the current system or present in trace amounts at best. Lactic acid production seemed generally lower for these organisms than in the bacteria considered to be standard fecal types. Acetic acid levels, in general, were similar to those of other coliforms. Texts refer (19, 102) to acetic acid being present in much lower concentrations than lactic acid.

The standard of reproducibility of results match that of other workers. Relative peak areas have been found to be reproducible and, with a few exceptions, examination of Tables VI-IX show the same to be the case here. Other workers have shown a wide variability between amounts of cell wall components or metabolic

products obtained from strain to strain of the same species (77, 79, 80, 90). Reproducibility of results has been found to be dependent upon extraction efficiency, volatility of the compounds extracted, detector sensitivity, uniformity of injection procedure, and age of cultures. The latter may not be such a factor here since spent cultures were routinely used and time studies showed results similar to those obtained with younger cultures. It was deemed important that the same organisms physiologically react the same in each new batch of media under the same conditions. Injection procedures would also seem to be important. Even with a sample size of 14 µl a slight variation of injection technique can produce large errors. In the extraction procedure, corks on the ether-containing tubes may pop resulting in loss of ether and possibly some of the volatile extracts.

The GC data was separated into the acids produced by a type such as -+-- or ++-- from a particular source, such as an elk, a horse, or water. Also all products produced by the isolates from a source were compared to discover if simply comparing the data of all the organisms isolated from various sources would not be simpler. Generally, while there was overlapping of values, various types from different sources as well as the total number of organisms from a particular source were found to produce different amounts of the respective

acids. The organisms from some sources, such as those from humans, showed no overlap of values in the lower and middle ranges of values with other organisms; although, there was some overlapping in the upper ranges. Statistical comparisons of these values by the t test (98) are given in Table XIII, the P value relationships are shown in Figures 12-13.

It is known that the canyon from which the bacteria were isolated is used primarily by an elk herd. A statistical analysis of levels of acetic acid produced by the standard fecal coliforms of elk (-+--, ++--) would indicate that the probability that the organisms of the water are related to elk coliforms is nearly 90%. Using the same acid, the relationship between the water -+-+ strains and the same isolates from elk is between 20 and 40%.

Comparison of the acetic acid levels of human fecal coliforms to the acetic acid levels of those found in water revealed that the probability of any E. coli here being from human fecal sources is nearly 0.001%. Comparison of the single human isolate of type -+-- against the mean of the water isolates of this type is about 0.05%. Data for the lactic acid levels show similar results indicating analysis of either acid would serve to separate human E. coli strains from those of elk, and apparently water coliforms deriving from run-off from areas utilized by these animals.

The lactic acid relationship between human E. coli and those from water is less than 0.001%. Figures relating elk to human strains are similar.

The Enterobacter strains from deer were compared to the fecal types from water at a probability of P = .10 and < .001 for acetic and lactic acids, respectively. Enterobacter from deer were the only group actually salvaged for the type --++ and we wanted to determine the relation between the unexpected amounts of acetic acid from this organism and that from regular fecal coliforms. The single E. coli var. I strain from deer, when compared to the mean of water fecal coliforms has a relationship of nearly P = .40 for both acetic and lactic acids. A good relationship also exists between the organisms of the -+-+ strains isolated from deer and water (Table XIII).

Organisms from horses seem to occupy a position between wild game and humans. Analysis of horse and human E. coli var. I acetic acid levels revealed the closest t values. The figure for lactic acid for the same association compares more to the relationship organisms from big game have to the human strains.

Calculations were done comparing the average amounts of acetic or lactic acid produced by the variety of coliform types isolated from elk or water to the average of these products obtained from human

strains. These calculations showed an even smaller likelihood that elk or water derived coliforms in this instance could be related to strains isolated from humans ($P = \leq .001$). The same type of comparison between elk and water coliforms revealed no change in the close relationship between the organisms from these two sources. It should be noted that the average of metabolic products tends to fall when the products of strains of other types are included. Since only -+-- and ++-- strains were available from humans, this relationship ($P = \leq .001$) may not hold if products from human strains of other types were included.

Similar relationships are seen in Table XIII for the coliforms from other sources. Coliform isolates from bear are closely related to those from elk and water, but appear different from those of deer or humans. Deer isolates continue to show the same divergence of association with other organisms from other sources that have been illustrated in the other methods of analysis. Statistical comparison of the metabolic products of these coliforms reveals low levels of association between them. The highest relationship is the probability of $.20 > P > .10$ for acetic acid and $.40 > P > .20$ for lactic acid to the organisms isolated from bear.

It would appear that isolation of coliforms by type may not be necessary. Rather a conclusion concerning the various sources may

be reached by simply comparing statistically the lactic or acetic acid averages of the coliforms derived from a source of pollution to known values. Considering the metabolic products of isolates generally, precludes the necessity of isolating sufficient organisms of a certain type for analysis.

SUMMARY

Fecal coliforms have long been regarded as the primary indicator of fecal pollution. Expanding recreational use of our mountain watersheds, evidence that the areas contain wild animals that serve as reservoirs of intestinal pathogens, and data that indicate present methods for detecting fecal contamination may be inadequate, strongly indicates that a better means of detecting fecal pollution is essential.

Recent isolation of a number of strains of coliforms from the waters of a mountain stream and from the droppings of local wild game in an area uninhabited by humans presented an opportunity to identify fecal pollution by source, identify a new method of doing so and possibly define new coliform indicators of fecal pollution in wild game. Isolates of human coliforms were included to discover whether these could be distinguished.

The research method involved defining the strains biochemically by tests selected to identify most of the organisms to at least the generic level, to identify groupings of organisms by IMViC type and source, to identify by the EC test the organisms of fecal origin, to determine whether certain strains isolated from the water are unique to a particular mammalian origin and determine by gas chromatography whether individual strains produce distinguishable metabolic products in MR-VP broth. Gas chromatography was done using a Beckman GC-2

chromatograph with a column packed with Resoflex-LAC-1-R-296 as liquid phase.

The results of the biochemical reactions demonstrated that water coliform isolates were related to certain groups of coliforms of mammalian origin. This was particularly true in the group of coliforms traditionally identified as fecal coliforms, Escherichia coli var. I and II. Overlapping of strains did occur, that is, organisms from more than one mammalian source could be placed in the same group. EC test results effectively identified fecal isolates from elk, but EC results from the stools of other species studied were variable; no EC positive isolates were obtained from deer.

The results of numerical analysis using the 20 assay results revealed no set patterns of similarity values for organisms of any particular source. Only an occasional isolate from deer appeared to be related to individual water strains.

The few coliform strains available from the fecal flora of deer were interesting in that none were EC positive, only one was identified as an E. coli, and most were similar to types commonly thought to occur in soil and on vegetation.

The results of gas chromatography of broth cultures revealed coliforms derived from various sources produced varying amounts of metabolic products. No bacterial fingerprints could be obtained but

statistical analysis of acetic and lactic acid, the major acids produced, revealed significant similarities between water and elk microorganisms ($.90 \geq P \geq .50$) and significant differences from the human strains ($.005 \geq P \geq .001$). Again strains from deer gave data indicating this species may have a unique fecal flora.

APPENDIX A

Table XIV. Biochemical reactions of selected strains.

Key to Symbols Used in Table

Reactions on MacConkey Agar:

+ = Pink colonies formed (lactose fermenting).
- = Clear colorless or other colonies formed - see text (non-lactose fermenting).
(+) = Fermentation of lactose appears to proceed slowly, colonies are light pink.
M = Mucoid, symbol also applies to the other media on which this characteristic appears.

Reactions on EMB Agar:

MG = Metallic Green
L = Lavender Colonies
P = Pink Colonies

Reactions on Kligler's Iron Agar:

A = Acid reaction, applies to slant, butt understood to be acid. Symbol also applies to those other reactions where it is found.
K = Alkaline reaction, butt understood to be acid unless otherwise stated.
G = Gas, symbol also applies to those other reacations where it is found.

Other reactions:

+ = Compound is utilized by, or characteristic is positive for, the strain shown.
- = Compound is not utilized by, or characteristic is negative for, the strain shown.
(+) = Compound is slowly utilized by, or characteristic is slowly positive for, the strain shown. Reaction requires longer than 48 hrs to occur.
$\frac{+}{F}$ = Slight clouding of KCN Medium.
F = Fermentative
O = Oxidative
GT = Growth occurred at 44.5°C but no gas was formed.
ND = Test not done.
* = Strain selected for gas chromatography.

Reactions of Type -+-- Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2317*	-	L	KG	-	F	-	-	+	-	-	-	-	-	-	-	+	+	+	ND	-	A	(+)	(+)	-	A	-	-	A
6280H	-	L	KG	-	F	-	-	+	-	-	-	-	+	-	-	+	(+)	+	-	+	AG	-	AG	-	-	-	-	-
6230E*	-	L	KG	-	F	-	-	+	-	-	GT	-	+	-	-	+	(+)	+	-	+	AG	AG	AG	AG	A	-	-	A
6549E	-	P	KG	-	F	-	-	+	-	-	-	-	+	-	-	+	+	+	-	+	AG	-	AG	-	-	-	-	-
2713	+	P	KG	-	F	-	-	+	-	-	GT	-	-	-	-	+	-	+	-	-	A	(+)	(+)	A	A	AG	(+)	A
2740*	+M	P	AG	-	F	-	-	+	-	-	GT	-	-	-	-	+	-	+	-	-	A	A	A	-	AG	AG	(+)	A
2750*	+M	P	AG	-	F	-	-	+	-	-	GT	-	-	-	-	+	-	+	-	-	A	A	A	-	A	AG	(+)	A
2934	+	P	AG	-	F	-	-	+	-	-	-	-	-	-	-	+	-	+	-	±	AG	A	AG	(+)	A	A	(+)	A
2943*	+M	P	KG	-	F	-	-	+	-	-	-	-	-	-	-	+	-	+	-	+	AG	A	AG	-	A	A	(+)	A
2708'	ND	ND	AG	-	F	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	A	A	A	-	AG	AG	(+)	A
6285E	+	PM	AG	-	F	-	-	+	-	-	-	-	+	-	-	+	(+)	(+)	-	-	AG	AG	AG	AG	-	-	-	-
2791*	+	P	KG	-	F	-	-	+	-	-	GT	-	+	-	-	+	-	+	-	-	A	AG	AG	-	AG	AG	(+)	AG
2852'	ND	ND	K	-	F	-	-	+	-	-	-	-	-	-	-	(+)	-	(+)	-	-	A	(+)	A	-	A	A	-	A
6178E*	+M	ND	KG	-	F	-	-	+	-	-	-	-	+	-	-	+	-	+	-	-	A	(+)	AG	-	A	A	(+)	A
2435*	+	MG	KG	-	F	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	A	+	A	A	A	A	-	(+)
6364H*	+	MG	AG	-	F	-	-	+	-	-	-	-	+	-	-	+	(+)	+	+	-	AG	AG	AG	AG	(+)	-	-	-
6156E*	+	MG	AG	-	F	-	-	+	-	-	+	-	-	-	-	+	(+)	+	(+)	+	AG	AG	AG	AG	AG	-	-	AG
6507E	+	MG	AG	-	F	-	-	+	-	-	+	-	+	-	-	+	(+)	+	+	-	AG	AG	AG	AG	-	-	-	-
2518*	+	MG	A	-	F	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A	-	A	A	(+)	-
2901	+M	MG	KG	-	F	-	-	+	-	-	-	-	-	-	-	(+)	-	+	-	-	AG	AG	AG	AG	AG	A	-	A

Reactions of TYPE -+--

Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
6304B	+M	MG	KG	-	F	-	-	+	-	-	-	-	-	-	-	+	-	+	-	±	AG	A	AG	-	AG	AG	(+)	A
6305B*	+	MG	K	-	F	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	AG	AG	AG	-	AG	AG	(+)	(+)
6306B*	+	L	KG	-	F	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	A	AG	A	-	AG	AG	(+)	A
6148B	+	L	KG	-	F	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	AG	AG	AG	-	A	AG	(+)	A
6176E*	+	L	KG	-	F	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	A	AG	AG	-	A	AG	(+)	A
2360	ND	LM	KG	-	F	-	-	+	-	-	+	-	+	-	-	-	-	(+)	-	+	A	(+)	AG	(+)	A	A	A	A
2098	ND	ND	AG	-	F	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	A	(+)	(+)	(+)	A	(+)	-	A
2444	+	P	A	-	F	-	-	+	-	-	GT	-	-	-	-	-	-	+	-	-	AG	(+)	A	-	A	AG	-	AG
2361*	ND	LM	K	-	F	-	-	+	-	-	GT	-	-	-	-	-	+	-	-	-	A	A	A	(+)	A	A	A	A
2430*	+	L	AG	-	F	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	AG	A	AG	A	A	-	(+)	A
2431	+	L	AG	-	F	-	-	+	-	-	-	-	-	+	-	-	-	-	(+)	-	A	A	(+)	A	A	-	-	A
6208D	+	L	AG	-	F	-	-	+	-	-	-	-	+	-	-	+	-	(+)	-	-	AG	A	AG	-	A	AG	-	A
6221D	+	L	KG	-	F	-	-	+	-	-	-	-	+	-	-	+	-	(+)	-	-	A	(+)	AG	-	A	AG	-	A
6034E*	+	L	AG	-	F	-	-	+	-	-	-	-	+	-	-	+	-	+	-	±	A	A	AG	-	A	AG	(+)	A
2433*	+M	L	KG	-	F	-	-	+	-	-	+	-	-	-	-	-	-	+	-	±	A	A	(+)	A	AG	-	-	(+)
6519E*	+	L	K	-	F	-	-	+	-	-	-	-	-	-	-	-	-	+	-	±	A	A	A	-	A	(+)	-	(+)
2434	(+)	L	KG	-	F	-	-	+	-	-	+	-	-	+	-	-	-	-	-	+	A	A	A	A	A	AG	-	(+)
2758	(+)	P	AG	-	F	-	-	+	-	-	+	-	-	+	-	-	-	-	-	ND	A	-	A	-	A	-	-	A

Reactions of Type -+--

Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
6392E*	+	L	KG	-	F	+	-	+	-	-	-	-	+	+	-	+	-	+	-	-	AG	AG	(+)	-	A	AG	-	AG
2449*	(+)	P	KG	-	F	-	-	+	-	-	GT	-	-	-	-	-	-	-	-	±	A	(+)	A	-	A	A	-	A
2611	+	L	KG	-	F	-	-	+	-	-	GT	-	+	-	-	-	-	-	-	-	AG	-	AG	(+)	(+)	(+)	-	(+)
6520E*	(+)	L	KG	-	F	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	A	(+)	AG	-	AG	AG	-	AG
2946	(+)	P	AG	-	F	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	AG	A	AG	AG	A	-	-	A
9-14035A*	+	MG	AG	-	F	-	-	+	-	-	+	-	+	-	-	-	(+)	+	+	±	AG	AG	AG	AG	-	-	-	-

Reactions of Type ++-- Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2204	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	AG	(+)	(+)	-	(+)
2148	+M	L	AG	-	F	-	+	+	-	-	-	-	-	-	-	+	-	+	-	-	AG	AG	AG	AG	(+)	-	-	-
2212	+	P	AG	-	F	-	+	+	-	-	+	-	+	-	-	-	-	+	-	-	AG	AG	AG	-	(+)	-	-	-
2249	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2250*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2251	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2252	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2292	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2314	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2457	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2458*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2519	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2660	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2248	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2308	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2309*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2310*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2311*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2312	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2313	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2553	+	L	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	-	-	AG	AG	AG	-	-	-	-	-
2560	+	L	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	-	-	AG	AG	AG	-	-	-	-	-
2653	+	L	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	-	-	AG	AG	AG	-	-	-	-	-

Reactions of Type ++-- Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2286*	(+)	L	AG	–	F	–	+	+	–	–	+	–	–	–	–	+	+	+	–	–	AG	AG	AG	s	–	–	–	–
2290	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	+	–	AG	AG	AG	AG	(+)	–	–	AG
2288	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	+	–	AG	AG	AG	AG	(+)	–	–	(+)
2295*	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	+	–	AG	AG	AG	AG	AG	–	–	AG
2298	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	+	–	AG	AG	AG	A	(+)	–	–	AG
2294	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	+	–	AG	AG	AG	AG	(+)	–	–	(+)
2297	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	+	–	AG	AG	AG	AG	–	(+)	–	–
2557	+	L	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	–	–	AG	AG	AG	–	(+)	–	–	–
2558	+	L	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	–	–	AG	AG	AG	–	(+)	–	–	–
2563	+	L	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	+	+	+	±	AG	AG	AG	–	–	–	–	–
2597	+	L	AG	–	F	–	+	+	–	–	+	–	+	–	–	(+)	+	(+)	+	–	AG	AG	AG	–	(+)	–	–	–
2599	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	+	–	AG	AG	AG	A	–	–	–	–
2645	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	+	±	AG	AG	AG	AG	–	–	–	–
6250E	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	+	+	±	AG	AG	AG	A	–	–	–	–
2291	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	–	–	–	AG	AG	AG	AG	(+)	–	–	–
2699	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	–	–	–	–	AG	AG	AG	–	(+)	–	–	–
2289	+	MG	AG	–	F	–	+	+	–	–	GT	–	–	–	–	–	–	–	+	–	AG	AG	AG	(+)	AG	–	–	(+)
2568	+	MG	AG	–	F	–	+	+	–	–	GT	–	+	–	–	–	–	–	+	–	AG	AG	AG	–	–	–	–	–
2647	+	MG	AG	–	F	–	+	+	–	–	–	–	–	–	–	–	–	–	+	–	AG	A	AG	–	–	–	–	–
2301	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	–	–	–	+	–	AG	AG	AG	–	–	–	–	–
2555	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	–	–	–	+	–	AG	AG	AG	A	–	–	–	–

Reactions of Type ++-- Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2562	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	AG	AG	AG	(+)	-	-	-	-
2648*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	-	-	-	+	-	AG	AG	AG	(+)	-	-	-	-
2650	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	-	-	-	+	±	AG	AG	AG	(+)	-	-	-	-
2554*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	-	-	-	ND	+	AG	AG	AG	AG	(+)	-	-	-
2561	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	-	(+)	-	ND	-	AG	AG	AG	(+)	-	-	-	-
2751	+	N	AG	-	F	-	+	+	-	-	+	-	+	-	-	-	+	-	-	-	AG	AG	AG	AG	-	-	-	-
2293	+M	P	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	+	+	+	-	AG	AG	AG	-	(+)	-	-	-
2300	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	+	+	+	-	AG	AG	AG	-	-	-	-	-
2303*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	+	+	+	±	AG	AG	AG	(+)	-	-	-	-
2304*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	+	+	+	±	AG	AG	AG	(+)	-	-	-	-
2319	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	+	+	ND	-	AG	AG	AG	(+)	-	-	-	-
2320*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	+	+	ND	-	AG	AG	AG	AG	-	-	-	-
6254H	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	+	+	+	-	AG	AG	AG	AG	-	-	-	-
6368H	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	+	+	+	-	AG	AG	AG	AG	(+)	-	-	-
6371H	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	+	+	+	-	AG	AG	AG	-	-	-	-	-
6372H*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	+	+	+	-	AG	AG	AG	-	-	-	-	-
6367H	+	MG	AG	-	F	-	+	+	-	-	-	-	+	-	-	+	+	+	+	-	AG	AG	AG	AG	-	-	-	-
6369H	+	MG	AG	-	F	-	+	+	-	-	-	-	+	-	-	+	+	+	+	-	AG	AG	AG	(+)	-	-	-	-
6370H*	+	MG	AG	-	F	-	+	+	-	-	-	-	-	-	-	+	+	+	+	-	AG	AG	AG	-	-	-	-	-
2296	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	ND	-	AG	AG	AG	A	-	-	-	-
2655*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	-	+	+	-	AG	AG	AG	A	-	-	-	-
2657	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	AG	-	-	-	-
2659	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	AG	-	-	-	-
2846	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-	AG	AG	AG	AG	-	-	-	-

Reactions of Type ++-- Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2451	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	-	+	ND	±	AG	A	AG	-	-	-	-	-
2456	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	-	+	ND	-	AG	AG	AG	AG	-	-	-	-
2547	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	-	+	ND	-	AG	AG	AG	A	-	-	-	-
6032E	+	ND	AG	-	F	-	+	+	-	-	-	-	+	-	-	+	-	+	+	±	AG	(+)	AG	AG	-	-	-	-
6390E*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	±	AG	AG	AG	AG	(+)	-	-	-
6295E	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	(+)	-	AG	AG	AG	(+)	-	-	(+)	-
2642	+	P	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	-	-	AG	AG	AG	AG	-	-	-	-
2654*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	+	+	±	AG	AG	AG	AG	-	-	AG	-
2665	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	(+)	+	-	AG	AG	AG	AG	-	-	AG	-
2299	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	-	+	-	AG	AG	AG	(+)	(+)	-	(+)	-
2427	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	-	+	-	AG	AG	AG	AG	-	-	AG	-
2302	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	+	+	+	±	AG	AG	AG	(+)	(+)	-	-	(+)
6506E*	+	MG	AG	-	F	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	AG	AG	AG	-	-	-	-	-
6389E*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	+	-	+	+	+	-	-	AG	AG	AG	-	-	-	-	-
2306	+	MG	AG	-	F	-	+	+	-	-	-	-	+	-	-	+	-	-	+	-	AG	AG	AG	-	-	-	-	-
2307	+	N	AG	-	F	-	+	+	-	-	-	-	-	-	-	+	-	-	+	+	AG	AG	AG	(+)	-	-	-	-
2549*	+	P	A	-	F	-	+	+	-	-	GT	-	-	-	-	+	-	-	+	±	A	(+)	A	-	A	A	(+)	(+)
6155E	ND	ND	A	-	F	-	+	+	-	-	+	-	+	-	-	+	-	-	+	+	A	A	A	A	-	-	-	-
2438	+	N	AG	-	F	-	+	+	-	-	+	+	+	-	-	+	(+)	-	-	-	AG	AG	AG	-	-	-	-	-

Reactions of Type ++-- Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2698	N	N	AG	–	F	–	+	+	–	–	+	–	–	–	–	+	(+)	–	+	–	AG	AG	AG	–	(+)	–	–	–
2664*	+	L	KG	–	F	–	+	+	–	–	–	–	+	–	–	+	+	–	+	–	AG	AG	AG	–	–	–	–	–
2569*	+	L	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	+	(+)	ND	+	AG	A	AG	AG	–	–	–	–
2613*	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	(+)	ND	–	AG	AG	AG	A	(+)	–	–	–
6252E*	+	MG	AG	–	F	–	+	+	–	–	+	–	–	–	–	+	(+)	(+)	+	–	AG	AG	AG	A	–	–	–	–
6318E	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	(+)	+	+	AG	AG	AG	–	–	–	–	–
6511E*	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	(+)	+	–	AG	AG	AG	AG	–	–	–	–
6256E*	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	(+)	+	(+)	(+)	+	+	AG	AG	AG	–	AG	–	–	AG
2432*	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	±	AG	AG	AG	AG	–	–	–	–
2564	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	–	AG	AG	AG	–	–	–	–	–
2652*	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	–	AG	AG	AG	–	–	–	–	–
2840	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	–	AG	AG	AG	(+)	–	–	–	–
2841	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	–	AG	AG	AG	(+)	–	–	–	–
2842	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	–	AG	AG	AG	(+)	–	–	–	–
6040E	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	±	AG	AG	AG	A	–	–	–	–
6100E	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	–	AG	AG	AG	A	(+)	–	–	–
6162E*	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	–	AG	AG	AG	A	–	–	–	–
6170E	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	–	AG	AG	AG	A	–	–	A	–
6247E	+	MG	AG	–	F	–	+	+	–	–	–	–	+	–	–	+	(+)	+	+	±	AG	AG	AG	AG	–	–	–	–
6248E	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	+	±	AG	AG	AG	A	A	–	–	–
6325E	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	(+)	+	(+)	+	+	+	AG	AG	AG	AG	(+)	–	–	–
6326E*	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	(+)	+	(+)	+	+	+	AG	AG	AG	AG	–	–	–	–
6508E	+	MG	AG	–	F	–	+	+	–	–	+	–	–	–	–	+	(+)	+	+	+	AG	AG	AG	–	(+)	–	–	–
6123E	+	MG	AG	–	F	–	+	+	–	–	+	–	+	–	–	+	(+)	+	–	–	AG	AG	AG	AG	–	–	–	–

Reactions of Type ++-- Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
6297E*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	+	AG	AG	AG	-	-	-	-	-
6499E	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	+	+	±	AG	AG	AG	AG	(+)	-	-	-
6501E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	(+)	+	(+)	+	(+)	-	AG	AG	AG	AG	-	-	-	-
6504E	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	(+)	+	(+)	+	+	-	AG	AG	AG	AG	-	-	-	-
6100'E	ND	ND	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	+	+	+	AG	AG	AG	AG	(+)	-	-	-
2567	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	ND	-	AG	AG	AG	AG	-	-	(+)	-
6384H*	+	MG	AG	-	F	-	+	+	-	-	-	-	-	-	-	+	(+)	+	+	+	AG	AG	AG	A	-	-	A	-
6028E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	(+)	+	(+)	+	+	+	AG	AG	AG	A	-	-	-	-
6249E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	+	AG	AG	AG	-	(+)	AG	-	-
6257E	+	N	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	+	AG	AG	AG	-	AG	AG	-	AG
6259E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	+	AG	AG	AG	-	(+)	-	-	AG
6292E*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	-	AG	AG	AG	A	-	-	-	-
6294E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	+	AG	AG	AG	A	-	-	-	-
6296E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	±	AG	AG	AG	-	-	-	-	-
6394E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	-	AG	AG	AG	-	(+)	-	-	-
6031E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	+	AG	AG	AG	AG	-	-	-	-
6293E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	(+)	+	(+)	+	+	±	AG	AG	AG	AG	-	-	-	-
6505E*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	(+)	+	(+)	+	+	-	AG	AG	AG	AG	(+)	-	-	-
2459*	N	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	+	+	-	AG	AG	AG	-	-	-	-	-
6298E	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	+	+	(+)	AG	AG	AG	-	-	-	-	-
6299E	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	+	+	(+)	AG	AG	AG	-	-	-	-	-
6300E	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	+	+	-	AG	AG	AG	AG	-	-	-	-
6328E*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	+	+	-	AG	AG	AG	-	-	-	-	-
6509E*	+	MG	AG	-	F	-	+	+	-	-	-	-	+	-	(+)	+	(+)	+	+	-	AG	AG	AG	AG	-	-	-	-
6493E	+	MG	AG	-	F	-	+	+	-	-	+	±	+	-	(+)	+	(+)	+	+	±	AG	AG	AG	-	-	-	-	-
6319E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	-	AG	AG	AG	-	-	-	-	-
2-2535A*	N	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	+	+	-	AG	AG	AG	-	±	-	-	-

Reactions of Type ++-- Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2747	+	P	KG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	+	+	+	AG	AG	AG	-	-	-	-	-
2933*	N	P	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	+	AG	AG	AG	(+)	-	-	-	-
14607AHu*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	+	AG	AG	AG	-	(+)	-	-	-
6362H*	+	MG	AG	+	F	-	+	+	-	-	-	-	+	-	-	+	(+)	+	+	-	AG	AG	AG	AG	-	-	(+)	-
6382H	+	MG	AG	+	F	-	+	+	-	-	-	-	+	-	-	+	(+)	+	+	+	AG	AG	AG	A	-	-	-	-
6388H	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	-	AG	AG	AG	AG	AG	-	-	(+)
6168E*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	(+)	-	AG	AG	AG	A	(+)	-	(+)	AG
6173E	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	+	+	-	AG	AG	AG	AG	-	-	-	(+)
6120E	+	MG	AG	-	F	-	+	+	-	-	+	-	+	+	-	+	(+)	+	+	-	AG	AG	AG	AG	(+)	-	-	-
6164E*	+	L	AG	-	F	-	+	+	-	-	+	-	+	+	-	+	(+)	+	+	+	AG	AG	AG	A	(+)	-	-	-
6321E	+	MG	AG	-	F	-	+	+	-	-	-	-	-	+	(+)	+	(+)	+	+	+	AG	AG	AG	AG	-	-	-	-
2500	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	(+)	(+)	+	ND	-	AG	AG	AG	-	-	-	-	-
2517*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	(+)	(+)	+	ND	-	AG	AG	AG	-	-	-	-	-
2548*	+	MG	AG	-	F	-	+	+	-	-	GT	-	+	-	-	(+)	(+)	+	ND	-	AG	AG	AG	A	-	-	-	-
6160E	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	(+)	(+)	+	ND	+	AG	AG	AG	A	(+)	-	-	-
2501	N	N	AG	-	F	-	+	+	-	-	+	-	+	-	-	(+)	+	+	ND	-	AG	AG	AG	-	(+)	-	-	-
2499	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	(+)	-	+	ND	-	AG	AG	AG	-	(+)	-	-	-
2598	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	(+)	ND	±	AG	AG	AG	AG	-	-	-	-
6313D*	+	L	KG	-	F	-	+	+	-	-	-	-	-	-	-	(+)	-	+	-	+	AG	AG	AG	-	A	A	(+)	A

Reactions of Type ++-- Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
6393E	+	MG	AG	-	F	-	+	+	-	-	-	-	-	-	-	+	-	+	+	-	AG	AG	AG	-	-	-	-	-
2656	N	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	-	-	+	-	AG	AG	AG	A	-	-	-	-
2755	N	L	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	-	+	-	AG	AG	AG	AG	(+)	-	-	-
6307B*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	-	+	-	AG	AG	AG	-	-	-	-	-
6289B	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	-	+	-	AG	AG	AG	-	-	-	-	-
6290B*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	-	+	-	AG	AG	AG	-	-	-	-	-
6361H	+	MG	AG	-	F	-	+	+	-	-	-	-	+	-	-	+	(+)	-	+	-	AG	AG	AG	A	-	-	-	-
6365H*	+	MG	AG	-	F	-	+	+	-	-	+	-	-	-	-	+	(+)	-	+	-	AG	AG	AG	AG	-	-	-	-
6383H	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	(+)	-	+	-	AG	AG	AG	AG	-	-	-	-
6159E*	+	MG	AG	-	F	-	+	+	-	-	-	-	-	-	-	(+)	-	+	+	-	AG	AG	AG	A	-	-	-	-
6253H*	N	N	KG	-	F	-	+	+	-	-	-	-	-	-	-	+	(+)	(+)	+	-	AG	AG	AG	-	AG	AG	-	-
6268H	-	L	K	-	F	-	+	+	-	-	-	+	-	-	+	-	-	-	-	+	AG	-	AG	-	-	AG	A	-
6277H	-	N	KG	-	F	-	+	+	-	-	-	-	+	+	-	-	+	+	+	±	AG	-	AG	-	AG	-	-	-
6202H	NM	N	KG	-	F	-	+	+	-	-	-	-	+	-	-	-	-	+	+	-	AG	AG	AG	-	AG	-	-	AG
6550E*	-	L	KG	-	F	-	+	+	-	-	-	(+)	+	-	+	-	-	-	-	+	A	-	-	-	-	-	-	-
6378E	ND	ND	K	-	F	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	AG	(+)	AG	-	-	-	-	-
4-17225AHu*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	(+)	+	(+)	+	+	+	AG	AG	AG	AG	-	-	-	-
5-2266AHu*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	(+)	+	(+)	+	+	+	AG	AG	AG	AG	-	-	-	-
8-1516Hu*	-	L	KG	-	F	-	+	+	-	-	GT	-	+	+	-	+	(+)	-	+	+	AG	(+)	AG	-	-	-	-	-

Reactions of Type ++-- Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
3-2229BHu*	-	L	KG	-	F	-	+	+	-	-	-	-	+	-	-	+	(+)	-	+	+	AG	-	AG	-	-	-	-	-
6-16974AHu*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	-	+	+	+	+	+	AG	AG	AG	AG	(+)	-	-	(+)
7-1940AHu*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	(+)	+	+	+	+	-	AG	AG	AG	AG	-	-	-	-
10-15431BHu*	+	MG	AG	-	F	-	+	+	-	-	+	-	+	-	(+)	+	(+)	(+)	+	-	AG	AG	AG	-	(+)	-	-	-

Reactions of Type -+-+ Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2243	+	N	AG	+	F	–	–	+	–	+	+	–	+	–	–	–	(+)	+	+	+	AG	AG	AG	–	–	–	–	–
6308B*	+	MG	AG	+	F	–	–	+	–	+	–	–	+	–	–	–	(+)	(+)	+	+	AG	AG	AG	–	–	–	–	–
6309B	+	MG	AG	+	F	–	–	+	–	+	–	–	+	–	–	–	(+)	+	+	+	AG	AG	AG	–	–	–	–	–
6146B	+	MG	AG	+	F	–	–	+	–	+	–	–	+	–	–	–	(+)	(+)	+	+	AG	AG	AG	–	–	–	–	–
6291B	+	N	AG	+	F	–	–	+	–	+	–	–	+	–	–	–	(+)	(+)	+	+	AG	AG	AG	–	–	–	–	–
6366H*	+	MG	AG	+	F	–	–	+	–	+	–	–	+	–	–	–	+	+	+	+	AG	AG	AG	–	–	–	–	–
6365H	ND	ND	AG	+	F	–	–	+	–	+	–	–	+	–	–	–	+	+	+	+	AG	AG	AG	–	–	–	–	–
6385E*	+	MG	AG	+	F	–	–	+	–	+	–	–	+	–	–	–	+	+	+	+	AG	AG	AG	–	–	–	–	–
6386E	+	MG	AG	+	F	–	–	+	–	+	–	–	+	–	–	–	+	+	+	+	AG	AG	AG	–	–	–	–	–
2287*	–	N	AG	–	F	–	–	+	–	+	GT	–	+	–	–	–	–	–	–	±	A	A	A	–	A	–	–	A
2429*	-M	L	KG	–	F	–	–	+	–	+	–	–	+	+	–	–	–	+	+	+	AG	AG	AG	–	AG	–	–	AG
2898	+	L	KG	–	F	–	–	+	–	+	–	–	+	–	–	–	–	(+)	+	+	AG	(+)	AG	–	A	A	–	A
6131E*	–	L	KG	–	F	–	–	+	–	+	–	–	–	–	–	–	–	(+)	+	+	AG	(+)	A	–	A	–	–	AG
2835*	NM	P	K	–	F	–	–	+	–	(+)	+	–	+	–	–	+	–	+	+	±	AG	AG	AG	–	AG	AG	(+)	AG
2838	N	P	KG	–	F	–	–	+	–	+	+	–	–	–	–	+	–	+	ND	±	A	AG	A	–	AG	AG	(+)	AG
2850	NM	L	KG	–	F	–	–	+	–	(+)	–	–	–	–	–	(+)	–	–	ND	+	A	(+)	AG	–	A	A	(+)	A
2906	(+)	L	K	–	F	–	–	+	–	(+)	–	–	+	–	–	(+)	–	(+)	ND	+	A	(+)	AG	–	A	A	–	A
61950*	+	L	KG	–	F	–	–	+	–	+	–	–	–	–	–	+	–	+	–	±	AG	AG	AG	–	A	AG	–	AG
6555'B	ND	ND	KG	–	F	–	–	+	–	+	–	–	+	–	–	+	–	+	–	±	A	AG	A	–	AG	AG	(+)	AG
6166E	+	N	KG	–	F	–	–	+	–	+	–	–	–	–	–	+	–	+	–	+	AG	AG	(+)	–	AG	A	(+)	A
6174E*	+	N	K	–	F	–	–	+	–	+	–	–	+	–	–	+	–	+	–	±	A	AG	AG	–	A	AG	(+)	A
6165E	+M	L	KG	–	F	–	–	+	–	+	–	–	+	–	–	+	–	+	–	+	A	A	AG	–	A	–	(+)	(+)
6165'E	ND	ND	KG	–	F	–	–	+	–	+	–	–	–	–	–	+	–	+	–	+	A	A	AG	–	(+)	(+)	(+)	(+)
2913	NM	P	K	+	F	+	–	+	–	+	–	–	+	–	–	+	–	+	ND	±	A	(+)	AG	–	A	A	(+)	A

Reactions of Type -+-+ Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2843*	NM	L	KG	-	F	+	-	+	-	+	-	-	-	-	-	+	-	+	-	+	A	A	AG	-	A	A	(+)	A
6311D*	+	MG	AG	-	F	+	-	+	-	(+)	-	-	-	+	+	-	-	-	-	-	AG	A	AG	A	A	-	-	A
6312D	+	MG	AG	-	F	-	-	+	-	(+)	-	-	-	+	+	-	-	-	-	-	AG	A	AG	A	A	-	-	A
2849*	+	L	K	-	F	-	-	+	-	(+)	-	-	-	-	-	-	-	-	-	-	AG	(+)	AG	-	A	A	-	A
2854	N	PM	KG	-	F	-	-	+	-	(+)	-	-	+	-	-	-	-	(+)	-	±	AG	(+)	AG	-	A	A	-	(+)
2951	-	P	KG	-	F	-	-	+	-	(+)	-	-	+	-	-	-	-	-	-	-	A	(+)	A	A	-	-	-	-
6154B	-	-	K	-	F	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	A	-	A	-	A	A	-	A
6136E*	+	N	KG	-	F	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	A	(+)	(+)	-	A	A	-	A
2948*	-	P	AG	-	F	-	-	+	-	(+)	-	-	-	-	-	(+)	-	(+)	-	+	A	A	AG	-	A	A	-	A
2072*	+	MG	KG	-	F	-	-	+	-	(+)	-	-	+	-	-	(+)	-	+	-	±	A	A	AG	AG	A	AG	-	AG
6314D*	+	MG	KG	-	F	-	-	+	-	(+)	-	-	-	-	-	+	-	+	-	+	AG	(+)	AG	-	A	AG	(+)	A
6315	+	L	KG	-	F	-	-	+	-	(+)	-	-	-	-	-	+	-	+	-	+	AG	A	AG	-	AG	AG	(+)	AG
6151H	±	MG	KG	-	F	-	-	+	-	+	-	-	-	-	-	-	-	-	(+)	-	AG	AG	-	A	-	-	-	-
6591D	(+)	P	KG	-	F	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	AG	(+)	A	A	A	-	-	-
6310D*	+	MG	AG	-	F	-	-	+	-	(+)	-	-	-	-	+	-	-	-	-	-	AG	AG	AG	A	A	-	-	A
6033'E	ND	ND	KG	-	F	-	-	+	-	(+)	-	-	-	+	-	(+)	(+)	-	+	+	AG	AG	AG	-	AG	-	-	AG
6030E*	+	N	KG	-	F	-	-	+	-	+	-	-	-	-	-	+	(+)	+	-	+	A	A	AG	-	A	AG	(+)	A

Reactions of Type --++ Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2215	(+)	-	AG	-	F	-	-	-	+	+	-	-	+	-	-	-	-	-	-	+	AG	(+)	AG	AG	A	-	-	A
2318*	-	L	AG	-	F	-	-	-	+	+	GT	-	+	-	-	-	-	-	-	+	AG	(+)	A	(+)	AG	-	-	AG
6194D	+	MG	A	-	F	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	A	A	A	A	A	-	-	A
2376	-	L	AG	-	F	-	-	-	+	+	-	-	+	+	-	-	+	+	-	+	AG	AG	AG	AG	AG	-	(+)	AG
6279H	(+)	L	AG	-	F	-	-	-	+	+	-	-	+	-	-	-	+	+	(+)	+	AG	AG	AG	AG	AG	-	-	-
2377	(+)	M	PM	KG	-	F	-	-	-	+	+	-	-	+	-	-	-	-	-	ND	+	AG	AG	AG	AG	AG	-	AG	AG
2381	(+)	M	PM	KG	-	F	-	-	-	+	+	-	-	+	-	-	-	-	-	ND	+	AG	AG	AG	AG	AG	-	AG	AG
2378	(+)	M	PM	KG	-	F	-	-	-	+	+	-	-	+	-	-	-	+	+	-	+	AG	AG	AG	AG	AG	-	AG	AG
2380	ND	PM	KG	-	F	-	-	-	+	+	-	-	+	-	-	-	+	+	-	+	AG	(+)	AG	AG	AG	-	AG	AG
2445	(+)	M	PM	KG	-	F	-	-	-	+	+	GT	-	+	+	-	-	-	+	-	+	AG	AG	AG	ND	(+)	-	-	-
6552D*	(+)	MG	KG	-	F	-	-	-	+	(+)	-	-	-	+	-	-	-	-	-	-	AG	A	AG	A	A	-	-	A
6554D	(+)	L	KG	-	F	-	-	-	+	+	-	-	-	+	-	-	+	-	+	+	AG	(+)	AG	-	AG	-	-	(+)
6593D*	-	L	KG	-	F	-	-	-	+	+	-	-	+	+	-	+	-	+	-	+	AG	-	AG	AG	A	-	(+)	-
6275H*	(+)	M	PM	KG	-	F	-	-	-	+	+	-	-	+	+	-	(+)	+	+	+	+	AG	(+)	AG	AG	AG	-	-	-
6281H	(+)	M	N	KG	-	F	-	-	-	+	+	-	-	+	-	-	-	+	(+)	+	+	AG	AG	AG	(+)	AG	-	-	-
6283H	(+)	M	PM	KG	-	F	-	-	-	+	+	-	-	+	-	-	-	(+)	(+)	+	+	AG	AG	AG	AG	AG	-	-	-
6594B*	(+)	N	KG	-	F	-	-	-	+	+	-	-	+	+	-	+	(+)	+	-	+	AG	-	AG	AG	A	-	(+)	(+)
6590E*	-	L	K	-	F	-	-	-	+	+	-	-	+	+	-	+	(+)	+	-	+	A	-	A	A	A	-	-	-

Reactions of Type --++

Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
6138'E	ND	ND	KG	-	F	-	-	-	+	+	+	-	+	-	-	(+)	+	+	+	+	AG	AG	AG	-	AG	-	-	AG
6196M*	(+)	L	KG	-	F	-	-	-	+	+	-	-	+	+	-	-	+	+	+	±	AG	(+)	AG	-	AG	-	-	AG

Reactions of Type -+++ Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2521*	(+)M	ND	KG	-	F	-	-	+	+	+	-	-	-	-	-	-	-	(+)	ND	+	AG	(+)	AG	AG	A	-	-	A
6138E*	(+)	L	KG	-	F	-	-	+	+	+	-	-	-	-	-	-	-	(+)	+	+	AG	AG	AG	-	AG	-	-	AG
2708*	(+)	P	KG	-	F	-	-	+	+	+	-	-	-	-	-	+	-	+	ND	+	AG	A	AG	A	AG	AG	(+)	A
2712	(+)M	PM	KG	-	F	-	-	+	+	+	GT	-	-	-	-	+	-	+	ND	+	AG	A	AG	A	AG	AG	(+)	A
2739	N	P	AG	-	F	-	-	+	+	+	GT	-	+	-	-	-	-	+	-	+	A	AG	AG	-	AG	AG	(+)	A
2746	+	N	AG	-	F	-	-	+	+	+	GT	-	-	+	-	-	-	-	+	-	A	A	AG	A	A	-	-	A
2838	ND	ND	KG	-	F	-	-	+	+	+	-	-	+	-	-	-	-	-	-	ND	AG	AG	A	-	AG	-	-	AG
6090E*	(+)	L	KG	-	F	-	-	+	+	+	-	-	-	-	-	-	(+)	(+)	+	+	AG	AG	AG	-	AG	-	-	AG
6517E*	(+)	L	KG	-	F	-	-	+	+	+	-	-	+	+	-	(+)	+	+	+	+	AG	A	AG	-	AG	-	-	(+)
Type -++-																												
2836*	(+)	P	KG	-	F	-	-	+	+	-	-	-	-	-	-	+	-	-	+	-	AG	AG	A	AG	AG	-	-	A
2758'	ND	ND	AG	-	F	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	A	A	A	A	A	-	-	-
6207D	+	MG	KG	-	F	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	AG	A	AG	A	A	-	-	A
6592D	(+)	P	KG	-	F	-	-	+	+	-	-	-	+	-	-	(+)	-	(+)	-	-	AG	(+)	AG	-	AG	-	-	-

Reactions of Type ++-+ Strain No.	MacConkey Agar	EMB	KIA	H_2S (KIA Agar)	O/F	Oxidase	Indole	Methyl Red	Voges-Proskauer	Simmons' Citrate	EC	Urea	Motility	Gelatin	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Mucate	KCN	Glucose	Lactose	Mannitol	Sucrose	Salicin	Adonitol	Inositol	Esculin
2315*	+	MG	AG	-	F	-	+	+	-	+	+	-	-	-	-	+	-	+	ND	-	AG	AG	AG	-	-	-	-	-
6267H*	-	P	KG	-	F	-	+	+	-	+	-	+	-	-	+	-	-	-	-	+	AG	-	AG	-	-	AG	(+)	-
6269H	-	P	K	-	F	-	+	+	-	+	-	+	-	-	+	-	-	-	-	+	AG	-	AG	-	-	AG	(+)	-
6270H	-	-	KG	-	F	-	+	+	-	+	-	+	-	+	+	-	-	-	-	+	AG	-	AG	-	-	AG	(+)	-
6543E	-	-	K	-	F	-	+	+	-	+	-	+	-	-	+	-	-	-	-	+	A	-	AG	-	-	AG	(+)	-
6582E	-	-	KG	-	F	-	+	+	-	+	-	+	+	-	+	-	-	-	-	+	AG	-	-	-	-	AG	-	-
6583E*	-	-	KG	-	F	-	+	+	-	+	-	+	-	-	+	-	-	-	-	+	A	-	-	-	-	AG	(+)	-
6584E	-	-	K	-	F	-	+	+	-	+	-	+	-	-	+	-	-	-	-	+	A	-	AG	-	-	AG	(+)	-
6585E	-	-	KG	-	F	-	+	+	-	+	-	+	+	-	+	-	-	-	-	+	AG	-	AG	-	-	AG	(+)	-
6586E	-	-	KG	-	F	-	+	+	-	+	-	+	+	-	+	-	-	-	-	+	AG	-	AG	-	-	AG	(+)	-
6587E	-	-	KG	-	F	-	+	+	-	+	-	+	-	-	+	-	-	-	-	+	A	-	AG	-	(+)	AG	(+)	-
6229E	-	ND	KG	-	F	-	+	+	-	+	-	+	+	-	+	-	-	-	-	-	A	-	(+)	-	-	A	(+)	-
Type +-++																												
2792*	(+)	P	AG	-	F	-	+	-	+	+	-	-	+	-	-	+	+	+	ND	+	AG	AG	AG	AG	AG	AG	AG	AG
6266H*	-	L	K	-	F	-	+	-	+	+	-	+	+	-	+	+	(+)	+	-	+	AG	-	AG	-	-	AG	(+)	-
Type ---+																												
2759*	+	L	AG	+	F	-	-	-	-	+	GT	-	+	-	-	-	(+)	+	-	+	AG	AG	AG	-	-	-	-	-
6197E*	NM	L	K	-	(F)	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	(+)	(+)	-	-	-	-	-	-
6225E	(+)	ND	K	-	O	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	(+)	-	-	-	-	-	-	-

APPENDIX B

Strain numbers assigned to groups used for computing similarity values.

(1) TYPE --+---

Group #	Strain #
1.	6280H
	6549E
2.	6230E
3.	2713
	2740
	2750
	2934
	2943
4.	6285E
5.	2791
	6178E
6.	6364H
7.	6156E
	6507E
8.	2901
	6304B
	6305B
	6306B
	6148B
	6176E
9.	2361
10.	2430
	2431
11.	6208D
11. (Cont'd)	6221D
12.	6034E
13.	2433
	6519E
14.	6392E
15.	2449
	2611
	6520E
16.	9-14034AHu

(2) TYPE ++--

1.	2249
	2250
	2251
	2252
	2292
	2314
	2457
	2458
	2519
	2660
2.	2248
	2308
	2309
	2310

(2) TYPE ++--

Group #	Strain #	Group #	Strain #
2. (Cont'd)	2311	13. (Cont'd)	2650
	2312		
	2313	14.	2561
			2751
3.	2553		
	2560	15.	2293
	2653		
		16.	2304
4.	2290		2309
	2288		
	2295	17.	6254H
	2298		
	2294	18.	6367H
			6369H
5.	2557		
	2558	19.	2296
			2655
6.	2563		2657
			2659
7.	2597		2846
8.	2599	20.	2451
	2645		2456
	6250E		2547
9.	2286	21.	6390
10.	2291	22.	2642
	2699		2654
			2665
11.	2289		
		23.	6506E
12.	2558		
	2647	24.	6389E
13.	2555	25.	2549
	2562		
	2648	26.	6155E

(2) TYPE ++--

Group #	Strain #
27.	2664
28.	2569
29.	2613
	6252E
	6511E
30.	2840
	2841
	2842
31.	6162E
32.	6325E
	6326E
	6508E
33.	6384H
34.	6257E
35.	6292E
36.	2459
	6728
37.	2747
	2943
38.	1-14607AHu
39.	6362H
	6382H
40.	6388H
41.	6168E
41. (Cont'd)	6173E
42.	6120E
	6164E
43.	6313D
44.	2656
45.	6307B
	6289B
	6290B
46.	6361H
47.	6159E
48.	6253H
49.	6277H
	6282H
50.	8-1516Hu
51.	3-2229BHu
52.	6-16974AHu
53.	7-1940AHu
	10-15431BHu

(3) TYPE -+-+

1.	2243
2.	6308B
	6309B

(3) TYPE -+-+

Group #	Strain #
2. (Cont'd)	6146B
	6291B
3.	6366H
	6365H
4.	6385E
	6386E
5.	2429
	2898
6.	6131E
7.	2835
	2838
8.	2850
	2906
9.	6195D
10.	6555B
11.	6166E
	6174E
	6165E (b)
12.	6312D
13.	2849
	2854
	2951
14.	6154B
15.	6136E
16.	6314D
16. (Cont'd)	6315D
17.	6151H
18.	6310D
19.	6030E

(4) TYPE --++

1.	2215
	2318
2.	6194D
3.	2376
4.	6279H
5.	2377
	2381
6.	2378
	2380
7.	6552D
8.	6554D
9.	6593D
10.	6275H
11.	6281H
	6283H
12.	6594B
13.	6590E
14.	6196M

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