FATTY ACID COMPOSITION OF BACTERIAL LIPIDS AS A MEANS OF DIFFERENTIATING SPECIES.

BY

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ABSTRACT

The fatty acid composition of twenty-seven bacterial strains, including seventeen different species of Corynebacterium have been studied. Fatty acids were extracted directly from whole washed cells and examined as methyl esters by gas liquid chromatography. The most abundant acid in four corynebacteria species was a C_{15}-saturated branched-chain acid. The fatty acid profiles of these four plant pathogens strongly resemble those of certain species of Propionibacterium and on this basis it was felt that these four species could be reclassified. The most abundant fatty acids of the remaining corynebacteria were hexadecanoic and octadecenoic acid.

Visual and quantitative comparisons of the chromatograms for the presence and relative amounts of large major peaks allowed rapid differentiation of corynebacteria into two major groups. Differences within each group were not as great, but significant enough to distinguish most species from each other.

The experimental method was evaluated and discussed as regards medium, period of bacterial growth, harvesting method, extraction solvent and analytical technique.

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"...The chymists are a strange class of mortals impelled by an almost insane impulse to seek their pleasure among smoke and vapour, soot and flame, poisons and poverty, yet among all these evils I seem to live so sweetly, that may I die if I would change places with the Persian King..."

Johann Joachim Becker,
"Acta Laboratorii Chymica
Novacencis, sen Physica
Subterranea" (1669)

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6	Hexane Blank		92
7	Reagent Blank		92
8	*C. fascians* after TLC		93
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10	Std. Mix D		94
11	Std. Mix F		94
12	Std. Mix BC-L		95
13	Std. Mix BC-1		95
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17	*C. pointsettiae*	CP-2	97
18	*C. michiganense*	CM-1	98
19	*C. flaccumfaciens*	CF-3	98
20	*C. creatinovorans*	7562	99
21	*C. renale*	6451	99
22	*C. xerosis*	373	100

23	C. xerosis	7711	100
24	C. fascians	CF-1	101
25	C. "Q"	149	102
26	C. pseudodiphtheriticum	10700	103
27	C. pseudodiphtheriticum	10701	103
28	C. pseudotuberculosis	1033	104
29	C. pseudodiphtheriticum	6981	105
30	C. bovis	3224	106
31	C. enzymicum	8155	107
32	C. diphtheriae	U 61 A	108
33	C. diphtheriae	U 61 B	108
34	C. hoagii	7005	109
35	C. equi	1621	110
36	C. ulcerans	7906	111
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38	C. xerosis	DL 7813	113
39	C. segmentosum	934	114
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47	C. pointsettiae	CP-2	117
48	C. michiganense	CM-1	118
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52	C. renale	6451	120
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INTRODUCTION

The mystery of bacterial evolution has resulted in the failure to produce a universally accepted natural classification of bacteria. An attempt at a phylogenetic classification was published by Bergey (12) in 1923, in order to stimulate efforts to perfect the classification of bacteria into orders, families, and genera as had been suggested earlier by a Committee of the Society of American Bacteriologists. However, this classification was never entirely satisfactory to authorities, as can be noted by comparing the sixth (1948) edition of Bergey's "Manual of Determinative Bacteriology" with the seventh (1957) edition (17). The sixth edition grouped bacteria into six orders containing 36 families while the latter has ten orders and 47 families. This revision was probably due, in part, to the fact that the characteristics of micro-organisms, upon which classification is based, are relatively few.

Early classifications were based on structural characteristics, particularly the shape of the cells. This had been found useful in drawing up natural classifications of higher plants and animals. However, as the number of known bacterial species increased, it was no longer adequate to know what the cells looked like, so it became necessary to consider what they did. Even this often failed to differentiate between certain species and so they were subgrouped according to their habitat or isolation source relationships and pathogenicity towards the host. This eventually led to the present mode of classification which is based on the following properties:

(a) Morphology:

Gross appearance of the culture,

Fine structure of the cell,

Staining properties

(b) Biochemical characteristics:

Optimal growth conditions

Fermentation and aerobic oxidation products

Reductions

Light absorption and emission

Exoenzyme production.

(c) Serology:

Specific antigen-antibody reactions.

(d) Toxigenic properties:

Exotoxin production

Endotoxin production.

Very often it was difficult to assign a certain organism to a definite category on this basis, since the cell shape might not be significantly different and the reactions, that it takes part in very limited. As a result, the search for better means of classification continued.

In 1956, Cummins and Harris (31) suggested that differences in composition of bacteria cell walls might be used to classify this form of life. In the same year, James and Martin (42) published a report describing the use of a new technique, gas-liquid chromatography, to determine fatty acids found in the culture media upon which bacteria had been grown. In 1961, Asselineau (7) suggested that GLC could be applied to the determination of bacterial fatty acids. Abel et al (1) in 1963, using gas-liquid chromatography, showed that different families of

one order have distinguishably different cellular fatty acid composition. Brown and Cosenza (20), in 1964, used lipids to distinguish between genera of bacteria, while Yamakawa and Ueta (86), in 1964, used carbohydrate, as well as GLC analysis of cellular fatty acids, to distinguish between species of Neisseria.

Research of bacterial lipids using gas-liquid chromatography, up to the present time has employed several approaches: some workers have analyzed chemical compounds extracted directly from cells (37, 68), others have separated out the membrane components and worked with these (79), some have confined themselves to the metabolic products of bacterial growth left in the media (63, 69, 19), workers have thermally decomposed the organisms and have studied the products of pyrolysis (77, 78, 36) while others have studied the bacteria by chromatographing the head-space vapors from milk (10).

Despite the amount of work done in this field, no one has attempted to differentiate all the species within a single genus. It is therefore our aim to study one genus in detail, with the hope that a simple and uniform technique might be developed for classification.

The genus Corynebacterium was selected for this study since, at the present time, classical bacteriological techniques have not been able to classify this genus adequately. This is probably due to the fact that, except for C. diphtheriæ, these organisms are quite inactive, hence hard to tell apart and relatively unimportant from a medical point of view, due to their low pathogenicity. In several instances, the differentiation has been based upon isolation source, rather than upon biochemical or morphological properties.

Thus, our study takes on the following objectives:

(a) to use the technique of gas-liquid chromatography to distinguish one species of Corynebacterium from another on the basis of fatty acid composition.

(b) to analyze the cellular fatty acid composition of one genus as completely as possible at this time.

(c) to shed some light on the controversy which enshrouds the classification of this genus.

This thesis is divided into four sections. The first section includes a review of basic microbiology as well as a literature survey. The concepts of microbiology presented here are elementary to a bacteriologist but provide basic information to a chemist without a background of microbiology. The secondsection lists the sources of the materials used and describes the method employed. The third section discusses the method as well as the experimental findings. The fourth and final section summarizes the work done and presents the conclusions.

HISTORICAL LITERATURE REVIEW

SOME BASIC CONCEPTS OF MICROBIOLOGY

The history of microbiology can be traced back to Antony van Leeuwenhoek (1632-1723) whose greatest claim to fame was not the invention of the microscope but rather the discovery of the microbial world (82). When scientists were able to see this "vast world of microscopic creatures" with their own eyes, the science of microbiology began to develop. Scientists soon wondered where these various forms originated. Some believed that microorganisms were formed spontaneously from the nonliving materials present in infusions, while others believed that the "seeds" or "germs" of these microscopic creatures were always present in the air, from which they could enter and grow under suitable growth conditions.

From numerous scientific reports it began to become apparent that spontaneous generation in plants and animals does not occur. However, it took the experiments of Pasteur and Tyndall, which showed that living matter does not arise de novo on our planet, to finally discredit the doctrine of spontaneous generation.

The second major scientific advance which accurred in the 19th century was the recognition of biological evolution. In 1859 Darwin published his book "The Origin of Species", and for the first time man saw life as a historical process which began with the evolution of our planet, and gradually developed more complex organisms to yield the various kinds we have today.

Meanwhile the quest for scientific clues leading to the primary origin of life on earth continued. Two scientific hypotheses to account for this existed. The first suggested that spores transported through space from some other

cosmic habitation infected the earth. This hypothesis has lost significance as a result of our increasing knowledge of outer space. The second hypothesis expressed the belief, that living matter arose from the mutation of non-living matter on the earth itself. This also seemed hard to accept since even the simplest microorganism is very complex in organization and chemical structure. Not until fairly recently have men been able to show that the latter proposal is a possibility. Haldane and Oparin (82) pointed out that before any microorganism existed on earth, any organic chemicals that might have been formed by chemical means would have possessed far greater stability than at present since there were no microorganisms to destroy them. They suggested that at an early time in the earth's history, a massive synthesis and accumulation of organic matter took place by photochemical reactions in the primitive atmosphere. This matter accumulated in the primitive oceans where it underwent subsequent chemical change to produce molecules of an increased complexity. Thus chemical evolution led to self reproduction which resulted in the formation of cellular organisms and eventually biological evolution. If one accepts this explanation and applies these ideas to microorganisms, it is to be expected that no clear cut distinction between many of the bacterial species would exist, but a great overlap in structural characteristics would result. It is not hard to see where the difficulty lies in attempts to classify bacteria by relating organisms to their evolutionary stage (phylogenetic classification) especially since until only recently, no supporting evidence has been obtained from fossilization.

Until about 1830, the living world could be readily divided into two different kinds of organisns: plants and animals. As explorations of the microbial world became more intensive, it became evident that many microbial groups were

difficult to fit into either of the two traditional groups. In the light of evolutionary ideas of today, this can be understood by thinking of the microbial groups as having branched off the pathway before the two other biological lines leading to plants and animals appeared.

To avoid the arbitrary assignment of transitional groups to one or the other kingdom, Haeckel, in 1866, proposed the establishment of a third kingdom, Protista. He distinguished members of this kingdom from true plants and animals, on the basis of their simple biological organization: they are unicellular, or if multicellular, do not show characteristically different regions of tissue. The finer details of their cellular structure could not be revealed by scientists until the electron microscope had been developed. This showed the existence of two different kinds of cells among organisms: a highly evolved type called the eucaryotic cell found in all plants, animals and in several groups of protists, and a simple type, the procaryotic cell. On this basis, the protists were divided into two groups (a) higher protists (with an eucaryotic cell structure) consisting of fungi, protozoa and most algae; (b) lower protists (with procaryotic cell structure) containing all bacteria and the blue-green algae.

The basic difference between the two types of cells lies in the fact that the eucaryotic cell nucleus is separated from the cytoplasm by a nuclear membrane and contains highly organized sub-units called chromosomes, while the procaryotic cell does not have its nucleus and cytoplasm clearly differentiated and is less highly organized. The sub-divisions of the Protista are shown in Fig. 1 on the next page.

The lower protists can readily be subdivided into blue-green algae

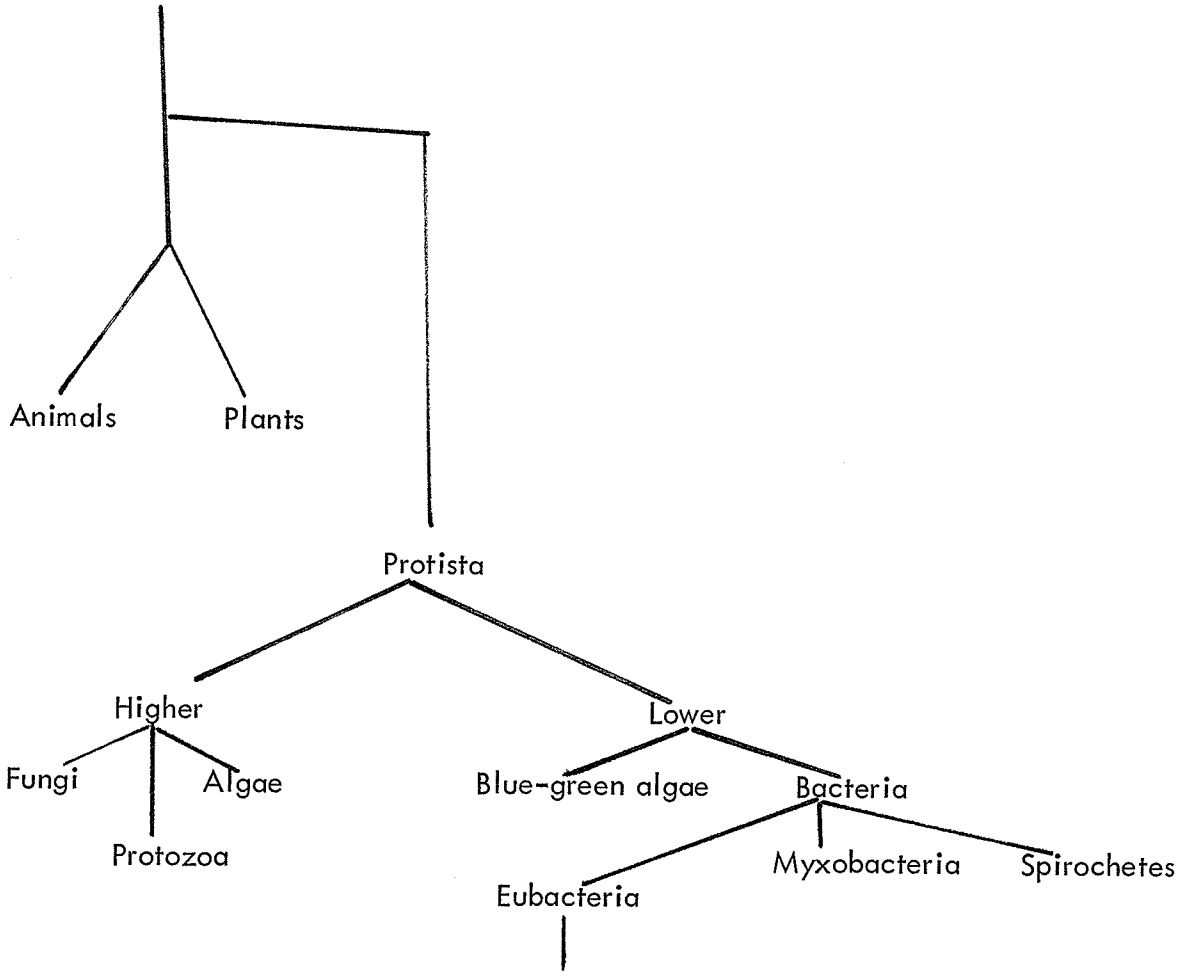

Fig. 1. Subdivisions of Protista.

and bacteria on the basis of certain distinctive characteristics common to the algae. The bacteria meanwhile can be further split into myxobacteria, spirochetes and eubacteria (or true bacteria) by considering their mechanism of movement and cell wall character. This brings us finally to the eubacteria which constitutes the largest and most diversified group of lower protists. Some of the basic structural characteristics in relation to chemical composition will be considered here, to provide some of the background upon which this research is based.

The presence of eubacteria was originally demonstrated by a differential staining procedure developed by Christian Gram in 1884 whereby bacteria are treated with crystal violet and iodine, followed by a washing in either acetone or alcohol. On this basis the "gram-positive" eubacteria turn violet and remain so after washing, while "Gram-negative" eubacteria become colorless after the solvent wash. While this was developed originally on an empirical basis, it became evident that the staining properties are correlated to some basic chemical difference of the cell. The difference was found to be in the cell wall: Gram-negative eubacteria have a high lipid content, while that of Gram-positive eubacteria is low (82). The cell wall, which is a protective structure designed to prevent the uptake of water in hypotonic solution to cause the cell to swell and burst, is mainly a macromolecular mesh composed of polysaccharides. In the lower protists, these polysaccharides are long chain polymers of simple sugars or amino sugars. Procaryotic organisms also have other chemical constituents in their cell walls such as lipids and proteins.

If one removes the cell wall enzymatically from Gram-positive bacteria, the existence of a cytoplasmic membrane may be demonstrated. Its

chemical composition is quite different from that of the cell wall, consisting mainly of protein and lipids with smaller amounts of RNA (82, 79). No membrane has been isolated from Gram-negative bacteria so far, due to difficulty in dissociating it from the cell wall.

The cytoplasm itself consists mainly of RNA found in the ribosomes. It may also contain reserve material such as poly-β-hydroxybutyric acid, polymerized inorganic metaphosphate or elemental sulphur.

The nucleus containing DNA has been discovered only recently, since nucleic acid staining resulted in the RNA of cytoplasm masking the DNA of the nucleus. Other parts of the bacterial cell are: (a) flagella -- long strands of fibrous protein always associated with "motility"; (b) capsules and slime layers -- organic polymers accumulated on the outer surface of some bacterial cell walls and usually composed of polysaccharides of varied composition; (c) endospores -- highly resistant bodies formed by some bacteria. The chemical structure is different from that of the original cell.

As can be seen from Fig. 1, page 9, eubacteria may be subdivided into many groups based upon a combination of shape, physiology and/or biochemical activities. This raises a difficult question in setting up taxonomic divisions, namely, which of these characteristics should be of primary importance. Should a group be established on the basis of similar physiological (or biochemical) activities while containing a variety of cell shapes, or should similar cell shapes be grouped containing a variety of physiological activities ?

This has caused a great confusion of

classifications to appear in the past. (25) but was resolved to an extent by Bergey's publication in 1923 of, "The Manual of Determinative Bacteriology" (12). The manual has gradually gained general acceptance in succeeding editions. Bergey attempted to establish a natural or phylogenetic classification in which organisms were grouped as much as possible according to the supposed degree of evolutionary relatedness. The order of precedence (in decreasing rank) as applied to the classification system is as follows:

Kingdom - Protista

Division - Lower protists

Class - Bacteria

Order - Eubacteria

Family - Corynebacteriaceæ

Genus - Corynebacterium

Species - (a) diphtheriæ

(b) xerosis

(c) pseudodiphtheriticum, etc.

A "species" has been defined as any primary subdivision of a genus and is considered by Cowan and Steel (30) as a basal unit. If a number of basal units containing similar characteristics are combined, we get the next higher unit, the genus; groups of genera combine to form a family, etc.

LITERATURE SURVEY

The family of bacteria we are mainly concerned with in this study are the Propionic acid and Corynebacteria (as Stanier (82) calls them) or Bergey's (17) Family XII, the Corynebacteriaceæ.

Stanier et al (82) give them this heading since propionic acid is produced in their metabolic products (as well as acetic acid, carbon dioxide and occasionally succinic acid). The corynebacteria can be distinguished from propionic acid bacteria by a physiological difference as may be shown by a comparison of their reactions (especially to oxygen) in Fig. 2, page 14 . They are club-shaped rods from which they derive their name: the Greek word (koryne) meaning a club and the Latin (Bacterion) a little staff (25). They are Gram-positive, rod-shaped bacteria that usually occur in palisade, V-form or cuneiform arrangements, as a result of their failure to separate completely during division. This peculiar mode of cell division is known as "snapping-fission".

The corynebacteria are a group of microorganisms characterised by their apparent lack of pathogenicity for man except for C. diphtherieæ which is notorious for its human pathogenicity. As a result, almost all the interest in the past has been concentrated on it while the remaining species of this genus have been left almost untouched. The disease diphtheria was recognized by Bretonneau (18) in 1826 but its cause was unknown. Two years later Trousseau (84) confirmed and extended Bretonneau's work. In 1883 Klebs (51) described the organism under the name of Microsporon diphthericum. However, he admitted having problems in obtaining pure cultures from diphtheritic membranes. Finally, however, in 1883 Löffler (58) showed that he could isolate the organisms in their characteristic arrangement from victims' organs, grow them on solidified blood serum and reproduce the disease experimentally in guinea pigs. The bacteria were given a specific rank by Trevisan between 1842 and 1890, but it was not until 1896 that Lehmann and Neumann (54) elevated the species of diphtheria and diphtheria-like organisms

	Propionic acid bacteria (Propionibacterium)	Coryneform bacteria (Corynebacterium)
Nature of energy-yielding metabolism	Propionic acid fermentation of sugars and usually of lactic acid	Respiration or sugar fermentation of lactic or propionic type
Relations to oxygen	Anaerobic	Aerobic; some facultatively anaerobic
Presence of cytochromes and catalase	+	+
Growth factors required (amino acids & vitamins)	few	few
Motility	absent	absent

Fig. 2. Distinguishing features of main groups of Gram-positive, non-spore forming rods.