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CHEMOTAXONOMY IN BACTERIAL SYSTEMATICS

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ABSTRACT. In taxonomy, polyphasic approach is based on the principle of combining and evaluating different types of data obtained from microorganisms. While, during characterization and identification of a microorganism, in the direction of polyphasic studies, chemotaxonomic analysis has of paramount importance for the determination of the most important differences between the family, genus and species comparatively. It is beyond doubt that, in recent years significant developments have been achieved in systematics by the aid of molecular biological studies. Phylogenetic data have revealed the hierarchical arrangement of the kinship relations between the given bacteria, however, this information cannot provide reliable data on the level of genus. At this stage, chemical markers play an important role in regulating inter-taxa relationships. Chemotaxonomy; is the whole of the characterizations made by using the similarities and differences of the biochemical properties of bacteria. In bacterial systematics, chemotaxonomy examines biochemical markers such as: amino acids and peptides (peptidoglycan), lipids (fatty acid, lipopolysaccharides, micolic acid and polar lipids), polysaccharides and related polymers (teicoic acid, whole sugar) and other complex polymeric compounds to find the distribution of members of different taxa and all of this information is used for classification and identification. In this review, how the chemotaxonomic data can be used in bacterial systematics and reflected to application within the field questions were evaluated.-REVIEW.

1. Introduction

Chemotaxonomy is defined as; the complete characterization made using the similarities and differences of the biochemical properties of bacteria. Chemotaxonomy examines the distribution of biological and chemical macromolecules containing; amino acids, peptides, lipids, polysaccharides and other related polymers, proteins, enzymes as well as, other complex polymeric molecules such as isoprenoid quinone and sterol among members of different taxa. Then, it uses this information for classification and identification [1, 27]. Phylogenetic data give a hierarchical skeleton of the relationship between bacteria but, it does not always provide reliable information when describing taxa of bacteria above the species level. On the contrary, chemical markers scattered irregularly in the taxa, may give information about the hierarchical levels of the taxa [2, 26, 27].

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Chemotaxonomic procedures are applied to determine the most important differences between species and strains comparatively in the direction of polyphasic studies. These procedures illustrate the separation of biochemical structures of the microorganisms thus, the properties of each species are explained using such analysis [3]. However, the studies are insufficient compared to the increasing number of microorganisms. Because of that, the role of chemotaxonomic analysis in the identification and characterization of species is of special importance.

A cell forms cell chemistry using a variety of building elements including cell membranes (fatty acids, polar lipids, respiratory lipokinons, pigments, etc) or cytoplasmic components in the outer cell layers (peptidoglycan, teicoic acids, micolic acids, etc). The general chemical properties of the cell comprise traditional chemotaxonomy. To compare new species characteristics, the chemotaxonomic properties of the nearest taxon should be investigated [27].

Chemotaxonomic methods have less specificity. Characterization based on bacterial chemical structure is not as consistent as genetic characterizations due to the variable structural features of bacteria. No matter how much the chemical structure of a bacteria display genetic characteristics, the environmental and breeding site conditions such as; chemical structure, osmotic pressure, pH, etc. can affect and change these characteristics [1, 26]. In general, bacteria are divided into two groups as gram positive and gram negative according to the chemical properties of their cell wall structure. When characterization methods are applied, taking into consideration of the differences between bacteria makes the application. In general, the characters used in bacterial chemotaxonomy are; peptidoglycan, diamino acids, polysaccharides, teichoic acids, mycolic acids, fatty acids, polar acids, isoprenoid quinones, polyamines, prokaryotic pigments and LPS.

2. PEPTIDOGLYCAN

The cell walls of Gram-positive bacteria are very diverse and the main reason behind this diversity is poly peptidoglycan. Peptidoglycan is a heteropolymer of glycine threads formed from cross-linked peptide bonds. The backbone of glycan is linked by a β-1,4 glycosidic bonds, consisting of alternating N-acetylglucose amine and N-acetyl muramic acid units. It is responsible for the protection of the cell wall rigidity, shape, and osmotic pressure against any defects. The polymer can be found in the cell walls of both Gram-positive and Gram-negative bacteria however, it is not found in the members of Archaea. Since the very first analysis,

peptidoglycan has been of great interest in the antibiotic and mechanisms of resistance researches, specifically in the behaviour of mobile phages, immune responses, identification and classification of bacteria. The discriminatory strength of peptidoglycan structure is limited by Gram-positive bacteria [4, 5].

Although the sugar molecules in the glycan structure are linked by β-1,4-glycosidic linkages which are strong bonds, it is insufficient in providing durability of the cell wall. Thus, the formation of cross-link between glycan chains provides full stabilization of the peptidoglycan structure [6].

Some differences found within the structure of peptidoglycan distinguish the two groups of bacteria as Gram-positive and Gram-negative. According to the differences in the cross-linking of the amino-sugar backbone, peptidoglycans can be divided into two groups; A-type peptidoglycan and B-type peptidoglycan [5].

3. Analysis of diaminopimelic acid

Diaminopimelic acid is found on the cell wall at the 3rd position. Structurally there are three forms; LL- , meso-, and OH-. They are named as; α- and ε- according to their different chemical structures. The presence or absence of these forms in Gram positive bacteria, particularly in Actinobacteria, separate them into different groups according to the type of diaminopimelic acid [7, 23].

The diamino acids in Gram (-) bacteria; including Spirochetes, with the exception of few groups of the Gram (-) bacteria, have a remarkably homogenous peptidoglycan structure. Here, the taxonomically significant diversity can be found in the peptide side chains, especially at the 3rd position. The most commonly distributed diamino acid in this position is, m-diaminopimelic acid, nonetheless, L-lysine is also quite common. The least common are; L-diaminobutric acid, LL-diaminopimelic acid and L-ornithine. In some bacteria at the 3rd position, diamino acid may be replaced by a monoamino acid such as; L-alanine or L-homoserine. For example; the peptidoglycans between Spirochetes usually contains m-diaminopimelic acid instead of L-ornithine. In many members of the Fusabacterium genus (Fusabacterium gondiaformans, F. necrophorum, F. nucleatum and F. russi) m-diaminopimelic acid is replaced with a sulfur analogue. In Fusobacterium mortiferum; L-lanthonine and m-diaminopimelic acid are both present. Therefore, although the taxonomic potential of the peptidoglycan may appear limited, it may be valuable for some bacterial groups. Thin Layer Chromatography (TLC) can be used in the analysis of diamino acids [8, 9, 27].

4. Sugar analysis

In the presence of DAP, *Actinomycetes* are groups according to the type of sugar they contain and are expressed in some chemotypes. In a study of a 16 *Actinomycetes*; four groups were identified according to the sugar content present in *Actinomycetes*. In group A, galactose and arabinose are present, xylose is absent. Madurose present in B group, but xylose or arabinose are absent. In group C, no identifiable sugar exists, however, xylose and arabinose present in group D. Thin Layer Chromatography (TLC) is used for the comparative analysis of sugars [9, 27].

TABLE 1. Sugar group in some *Actinomycetes*.

Cell wall chemotype	Type of Sugar	Organisms
I	-	Streptomyces, Streptoverticillium, Chainia, Actinopycnidium, Actinosporangium, Microellobosporia
II	D	Actinoplanes, Amorphosporanigum, Ampullariella, Dactylosporangium, Micromonospora
III	B	Actinomadura, Microbispora, Streptosporangium, Spirillospora, Planomonospora, Dermatophilu
	C	Thermoactinomyces, Actinobifida, Geodermatophilus
IV	A	Mycobacterium, Nocardia, Micropolyspora, Pseudonocardia, Thermomonospora

5. Teichoic acid

In gram positive bacteria cell wall, the polymer backbone contains various sugars and glycopolymers such as; phosphate, alanine, succinate, pyruvate, chlorine or mycolic acid binds together via diester bonds forming, teichoic acid [10].

Teichoic acid is located on the surface of the peptidoglycan by establishing a phosphodiester bond between; the peptidoglycan's N-acetylmuramic acid and teichoic acid's N-acetyl mannose amine. The lipoteichoic acid binds to the membrane of the glycolipids via phosphodiester bonds [10].

Teichoic acid is deposited on cell surfaces in order to interact with bacteria and other microorganisms, plants, proteins, and antibodies. Teichoic acids are negatively charged and play role in the formation of negative electrical charge found on the surface of gram-positive bacteria that extends outwards from the cell wall. In addition, it is also believed that teichoic acids may also play a role in the effective passage of ions through cell walls due to their negative charge. Teichoic acids are also involved in the formation of biofilms on biomaterials [10].

Teichoic acid is formed through recurring units of uronic acid, pyruvyl or succinyl groups. Furthermore, sugars blocks such as; glycerol, ribitol, erythrol, mannitol, arabinol and galactose form the main chain. Chain-polyol; is the main teichoic acid chain bearing many glycopolymers (sugars and non-sugar compounds). Polyol groups are separated into different types in accordance to the sugars and repetitive units it may contain. The distinction is made at the family and genus levels in bacteria. The type of teichoic acid found on the cell wall is of importance, as it serves as a marker in the distinction between Staphylococcus and Micrococcus species.

Teichoic acids are divided into five different groups according to the difference in the chemical composition of the polyol groups;

I-Polyolteichoic acid type I: (alditol-phosphate) glycerol, ribitol, erythrol, mannitol, arabinitol and their phosphodiester bonds may vary among species. *Staphylococcus aureus* [11].
II-Polyteichoic acid type II: (glucose-alditol-phosphate); there is a glycosyl group in the main chain of the teichoic acid.
III-Polyteichoic acid type III: (sugar-1-phosphate-aldethol-phosphate); in this type of teichoic acid, unsaturated bonds are formed between glyceric acid and acyl components. The dominant sugar in this type is N-acetyl hexosamine. *Bacillus subtilis* [12].
IV-Polyteichoic acid type IV: (sugar-1-phosphate-polyol phosphate); glycerol phosphate and sugar-1 phosphate are linked to the teichoic acid chain; it is used in the detection of Staphylococcus strains. The N-acetyl hexosamine is the dominant sugar [11].

V-Poly teichoic acid type V: (polyol phosphate); this type of teichoic acid contains; glycerol phosphate and glucose-glycerol-phosphate and it is used in the identification of Nocardiopsis strains.

Different species of the same genus may exhibit some differentiations, according to the type of teichoic acid it contains. For example; *Staphylococcus auresus* contains an N-acetylglucose amine ribitol teichoic acid. Whereas, *Staphylococcus saparophyticus* and *Staphylococcus xylosus* contain sugars such as; glucose or N-acetyl galactose amine glycerol teichoic acid [13].

6. Micolic acid

Micolic acid is generally found in *Nocardiaform actinomycetes* such as; Mycobacterium, Gordonia, Nocardia, and Rhodococcus and it is located between the lipid-rich cell walls and they provide resistance to acids. The acid is formed from α-alkyl side chains and β-oxy basic chains. Micolic acids are differentiated by the α-alkyl side chains and the long hydrocarbon chains that contain C atoms ranging from 20 to 90 atoms [1].

The presence of micolic acid increases the bacterial resistance to chemical changes and dehydration. Furthermore, it also permits an easy bacterial growth as well as increases the bacterium resistance to pathogens. Because of these properties, micolic acids are linked to hydrophobic antibiotics [14]. *Mycobacterium tuberculosis* strains produce 3 basic types of micolic acids; alpha-, methoxy-, and keto-. The alpha-micolic acid constitutes for the 70% of all micolic acids in the cell whereas, keto- and methoxy- micolic acids constitute only 10-15% of the micolic acids in the cell [1].

The number of C atoms in micolic acids varies according to the species and thus, it is used as a chemotaxonomic marker. The difference in C atom numbers is important in the differentiation of species and genus. For examples, when Nocardia, Mycobacteria, and Rhodococcus species are compared; the number of C atoms in micolic acids in Nocardia genus ranges from 42 to 58 whereas, in Mycobacterium genus the C atoms ranges from 70-90 atoms. However, in Rhodococcus it ranges from 36-46 C atoms. When the bacteria are examined at the species level; *Nocardis flovorosea* shows a range of 50-56, *Nocardia asteriodeste* has 46-58 while, *Nocardia crasostrea* has 42-56 C atoms. Consequently, micolic acid is a useful aid to make differentiation between both genus and species level [14, 24].

7. Fatty acids

Fatty acids are carboxylic acids with long aliphatic chains. Some examples of its chemotaxonomic varieties include hexa decanoic acid, sucro-propane fatty acids, iso-fatty acids, and anti-iso fatty acids. Fatty acids are considered as important chemical characters used to describe the members of a species and genus. As a chemotaxonomic character, hydrophobic lipids of the side chains and other lipid derivatives formed by these chains are used [1].

In the species of the genus Legionella a highly differentiated fatty acid strains exists and can be used to characteristically represent the species. Polyunsaturated fatty acids are rare but can be found in bacteria that grow in low temperatures and also present in cyanobacteria. Some fatty acids derivatives are commonly found in different large bacterium groups, these large groups are; alpha, beta and gamma Proteobacteria. Fatty acids may be observed in many different structural branching. For example, some species of Alicyclobacillus, Sulfobacillus, and Curtobacterium are known to have fatty acid varieties such as cyclohexane and cycloheptane [2].
Fatty acids are divided into five different groups according to the different chemical structures of the groups [25].

Hydroxy fatty acids: they are fatty acids derivatives; the hydroxyl groups can be observed to be attached to one or more C atoms. Examples are Azotobacter, Bacillus and Micrococcus.

Branched chain fatty acids: the hydroxyl or methyl groups are attached to one or more C atoms, in the parent chain. Examples are *Bacillus polymyxa, Sarcinia spp., Bacillus subtilis.*

Cyclopropane fatty acids: one or more incorporated cyclopropane rings can be observed along the fatty acid chain. Examples of bacteria in this group are *Agrobacterium tumefaciens, Escherichia coli, Clostridium butycrium.*

Unsaturated fatty acids: double bonds are formed along the main chain, it is not a very common fatty acid derivative, however, it can be observed in bacteria such as *Brucella suis,* Corynebacteria spp*.,* and *Escherichia coli.*

Fatty acids can be analysed using Thin Layer Chromatography (TLC), Colum Chromatography and Gas Chromatography [2].

8. Polar lipids

Polar lipids are only associated with cell membranes; they are lipid derivatives and are not limited to phospholipids. The most common ones are phospholipids which are phosphatidic acid derivatives (phosphoglycerides). Although the general pattern of polar lipids is homogeneous nevertheless, they are an important chemical characteristic used in the differentiation between families and species [1].

The simplest form are the acylglycosides, in which a mono- or oligosaccharide is esterified by a long chain fatty acid. 1,2 diol are a rare form of polar lipids that can be observed in both *Thermomicrobium roseum* and *Chloroflexus aurantiacus*. Polar lipids are rarely found in Archaea. For instance; *Thermoplasma acidophilum* phospholipids contain glucose and mannose [1, 15].

9. ISOPRENOID QUINONES

Isoprenoid quinones are vitamin-like structures found in the prokaryotic cell membranes. They are formed from isoprene subunits. It is found in both aerobic and anaerobic organisms. Structurally there are two main classes; benzoquinones and naphthoquinones (Menaquinones). Isoprenoid quinones that are found on the membranes of almost aerobic and anaerobic all organisms usually act as an electron carrier or antioxidants. The structural variation of the quinones, the differences in hydrogenation length as well as the differences in the polyprenyl side chains carry an important value in classification. Quinones are used as an important chemotaxonomic marker in the classification of the genus and family. Isoprenoid quinones are free lipids that can be easily removed from bacterial cells, using lipid solvents such as chloroform, acetone, or hexane [1].

The natural composition mixture of bacterial quinones can be easily identified using various chromatographic techniques. It is sensitive to photooxidation in the presence of oxygen and light. Gram positive bacteria only synthesize menaquinones or dimethylquinones, but they never synthesize ubiquinones. Facultative anaerobic Proteobacteria groups such as *E. coli* have both MQ and UQ. The low oxygen pressure increases the levels of menaquinonein in some facultative organisms, while the levels if ubiquinone are reduced [1, 16].

9.1. *Benzoquinones*

The general formulas for benzoquinones are as follows; 1,4-benzoquinons (ubiquinones) and 1,2-benzoquinones. They are also called as, ortho- and para-benzoquinones.

The differentiation between Q-8, Q-10 and so on is as a result of the isoprenoid subunits. The predominant ubiquinone Q-10 has 10 isoprenoid subunits.

Some members of the *Alfaproteobacteria* class have dominant ubiquinones Q-10 nonetheless; some of the taxa may also have Q-9 and Q-11 ubiquinones. *Betaproteobacteria* members usually have Q-8. While members of *Gamaproteobacteria* have a wide verity of ubiquinones. Moreover, in some groups rhodoquinones are synthesized. The majority of aerobic gram-negative bacteria produce only ubiquinones [1].

9.2. *Menaquinones*

Menaquinones are vitamin K derivatives. They are oil soluble and are divided into two groups; K vitamin 1 (phylloquinone) and K vitamin 2 (Fitonadion). In Bacteria and Archaea, the most important forms of menaquinones synthesized are MQ-11, MQ-7, and MQ-4. Aerobic respiration comes to life through menaquinones [16, 17].

10. POLYAMINES

Polyamines are chemical structures that occur following the formation of many amine groups from different conformations. The general types used in chemotaxonomy are 1,3-diamino propane, putrescine, 2-hydroxy putrescine, cadaverine, sym-norspermidine, spermidine, sym-homospermidin, and spermin. They can be found in prokaryotes, however; their concentration is significantly low. As a result, the prognosis when chemotaxonomy is used can be relatively low. Nevertheless, when classification is made, the dominant polyamine pattern in the group can be observed [1, 18].

Recent studies have shown that, polyamines may interact with DNA through specific or non-specific structures as well as stabilize conformation. In addition, polyamines provide an osmotic shock response that leads to an increase in the intracellular bacterial inorganic cations. This reaction is achieved by discharging polycationic ions.

Intracellular polyamines of some halophilic species may vary depending on the amount of salinity of the growth medium. This group can be exemplified with bacteria such as *Vibrio alginolyticus, Vibrio parahaemolyticus, Halomonas marina,* and *Rhodothalassium salexigens* [19].

In the Beneckea species unlike species of *Vibrio*, three unsaturated triamine sym-norspermidine residues have been observed. This observation suggested that Benekea species should be separated from *Vibrio* species. Other species of *Vibrio* have been characterized by the presence of sym-norspermidine. Aeromonas, Plesiomonas and two other *Vibrio* species also display this polyamine in their structure. It is worth mentioning that, *Vibrio fisheri* and *V. costicola* have been reclassified into the genus; Aliivibrio and Salinivibrio, respectively. The results of the aforementioned investigation indicate that the sym-norspermidine polyamine is a common feature of the genus Vibrio [19].

11. Prokaryotic pigments

Pigments are compounds that colours the cells. In many prokaryotic organisms, a wide range variety of colours can be observed in their cultures and colonies. These colours may be; yellow, red, purple, green, and many other different colours. Some of the pigments used in chemotaxonomy includes; lutein, cryptoxanthin, phytocyanin, zeaxantin (betacarotene), chlorophyll A and chlorophyll B.
Pigments can also be used as a chemotaxonomic marker. In rare cases, characterisation is applied in accordance to the colours given by the pigments. For some groups, pigments are of significance to their characterization. For example; anoxigenic phototrophic bacteria [1].

12. Lipopolysacchrides

The chemical complex of lipopolysaccharides (LPS) found on the outer membrane of Gram (-) bacteria are composed of different polysaccharide (PS) and lipids (Lipid A) parts. Most of their biological effects are caused by a lipid and the polysaccharide moiety that acts as an antigen for the actual cell. LPS is the most important component of the outer membrane. It is a major contributor to the structural integrity of gram (-) bacteria, and serves in the protection against chemical attacks on the cell. The lipopolysaccharide structure is generally comprised of three parts; O-antigen or chain, oligosaccharide nucleus and lipid A. Some properties of the inner nucleus are preserved among the taxa. Many bacterial

LPS nucleus contain; carbohydrates, phosphates components, amino acids, and ethanolamine. The polysaccharide of major surface antigens is called O-antigen. O-antigen or O-chain varies among different bacteria [5].

13. Conclusion

Chemotaxonomy provides an important solution for the identification of bacterial species so chemotaxonomic analysis should be mandatory to identify new species. In the past, when chemotaxonomic classification was not yet to be fully understood, many species were classified and published. The species classified during this period have to be reclassified and identified as they might belong to different groups. Astriking example is that, most of the bacteria belonged to the genus Thermomonospora and Microtetraspora at the beginning of 1998, were later identified as members of the genus Nonomuraea and were reclassified with the help of chemotaxonomic analysis [20, 21, 22].

No matter how much the chemical structure of the bacteria is present in their genetic architecture, the structure may be affected by the feeding site, osmotic pressure, pH, etc. as well as other environmental conditions. A standardized developmental procedure should be applied for the development of cultures, prior to a comparative chemotaxonomic study. Standardized culture conditions are of great importance in studies involving the qualitative analysis of chemical data [23]. As a result; in addition to molecular analyses and numerical studies, chemotaxonomic markers are important in the biochemical structure of living bacterial organisms so in their identification.

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