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Research Article

Study of Mycolic acid of genus *Microbacterium* and related strains

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Abstract

Aim: The aim of this study is to compare pattern of mycolic acid of bacterial isolates from dairy product to that of the type species of the genus *Microbacterium*.

Method: Thirty three bacterial isolates and reference strains were received from public and private culture collection. Extraction was performed by acid methanolysis procedure of freeze dried cells and samples of hexane extracts were spotted on thin layer plates coated with silica gel H and developed in petroleum ether.

Results: Out of thirty three strains tested for mycolic acid content only four strains demonstrated the content of mycolic acid. These strains were C90 (*Mycobacterium flavum*) and the unidentified strain BL77/18, BL 77/19 and BL77/20. The chromatographic mobility of mycolic acid of these strain resembled members of genus *Corynebacterium, Microbacterium liquefaciens* and *Microbacterium lacticum* failed to reveal any mycolic acid.

Keywords: *Microbacterium; Mycolic Acids; Methanolysis; TLC*

Introduction

Biological system are composed of multitude of different compounds and although there are components that are more or less ubiquitous some component show more restricted distribution. Researchers in the past pioneered the chemotaxonomic studies of bacteria by establishing the chemotaxonomic value of the chemical composition of the cell- walls of gram positive bacteria. The method has been subsequently extended to elucidate a variety of taxonomic difficulties. Although the simple fatty acids have so far proven to be of little direct taxonomic value, the more complex fatty acid referred to as mycolic acid showed important differences between various taxa and for routine screening of some species as *Mycobacterium* spp. [1]. The significant of mycolic acid in *Mycobacterium* survival and hence targeting the developing of anti-*Mycobacterium* agents was reported by Daffe M., *et al* [2]. Mycolic acids are exceptionally long-chain fatty acids that compose major and specific components of the cell envelope on member of the Corynebacteriales order [3-7]. Although the simple fatty acids have so far proven to be of little direct taxonomic value, the more complex fatty acids, usually referred to as mycolic acids, show important differences between various different taxa. Mycolic acids are generally agreed to be confined to the taxa of *Bacterionema, Corynebacterium sensu stricto, Mycobacterium, Nocardia, Rhodococcus* and *Micropolyspora brevicatena* [4]. Although the presence or absence of mycolic acids (or related alcohols and ketones) is of taxonomic significance, the variation in mycolic acids structure provides information of even greater value. The mycolic acids of the genus *Mycobacterium* are the most complex, usually containing between 60 - 90 carbon atoms and frequently exhibiting methyl branches, cyclopropane rings, and keto-, methoxy- or carboxy-groups acids which contain 36 - 66 carbon atoms and have 0 - 4 unsaturated links in the fatty acid side-chain [4]. In contrast, the mycolic acids of coryneform bacteria are relatively simple, containing between 20 - 36 carbon atoms and at most, a single double bond in the fatty

acid alkyl group [7-12]. *Corynebacterineae* are characterized by the presence of long chain lipid notably mycolic acid (α-alkyl β-hydroxy fatty acid) the structure of which are genus-specific [13]. The available data on the presence of absence of free or bound mycolic acid in coryneform and related bacteria has been summarized [14]. A generalized structure of mycolic acid is shown in figure 1.

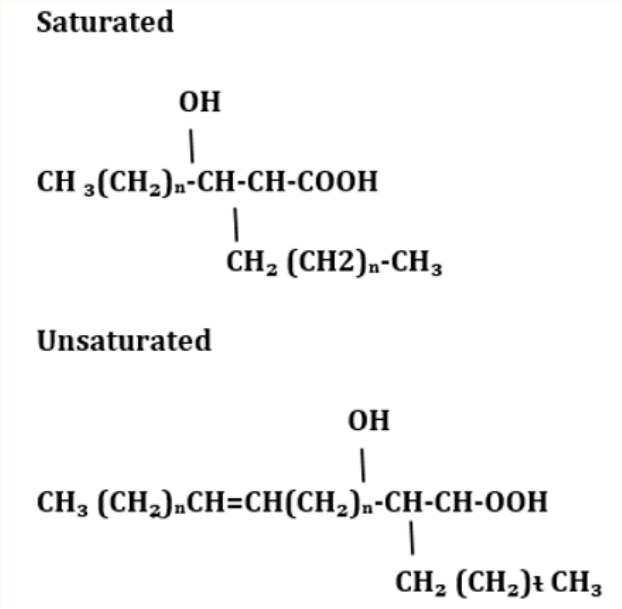

Figure 1: *Mycolic acids structure.*

Method

Collection of strains

Thirty tree strains of coryneform and related bacteria were collected for the study. These were obtained from public and private culture collections (Table 1). Each strain was given number for ease handling. A number of strains representing different taxa defined in study [15,16] where numerical phonetic surveys of coryneform and related bacteria were included as references. The unidentified strains were received from the National Institute of Research in Dairying, Sheffield, Reading and food Research institute, Norwich. All strains were originally obtained in freeze dried cultures.

Lab No	Species	Mycolic acid
C88	*Microbacterium lacticum*	-
C89	*Microbacterium lacticum*	-
C786	*Microbacterium lacticum*	-
C787	*Microbacterium lacticum*	-
C788	*Microbacterium lacticum*	-
C789	*Microbacterium lacticum*	-
C769	*Microbacterium liquefaciens*	-
C770	*Microbacterium liquefaciens*	-

C771	*Microbacterium liquefaciens*	-
C90	*Microbacterium flavum*	+
12	Unspecified	-
17	Unspecified	-
32	Unspecified	-
40	Unspecified	-
41	Unspecified	-
42	Unspecified	-
44	Unspecified	-
C315	Unspecified	-
C320	Unspecified	-
C321	Unspecified	-
JP2/1/1	Unspecified	-
JP2/1/4	Unspecified	-
JP2/1/15	Unspecified	-
JP2/1/21	Unspecified	-
JP2/1/3	Unspecified	-
JP2/1/6	Unspecified	-
JP2/1/11	Unspecified	-
JP2/10/6	Unspecified	-
JP2/6/3	Unspecified	-
JP2/6/5	Unspecified	-
BL77/18	Unspecified	+
BL77/19	Unspecified	+
BL77/20	Unspecified	+

Table 1: *The presence of mycolic acid in different stains.*

General growth media

Strains were routinely subculture at three weeks intervals onto nutrient agar (Difco) plates and stored at 4°C. after 2 - 3 days incubation at 37°C. For broth culture Nutrient broth Media (Oxoid) media was and culture were stored at lyophilized aliquots in glass vials for future used were they resuscitated by transfer small amount of nutrient onto nutrient agar plates and incubated for 7 days. Nutrient agar (Difco) and nutrient broth (Oxoid) were used for growing all isolates. All media were sterilized by autoclaving at 121°C for 15 minutes. Culture were incubated for 24 - 48 H at 37°C. Cultures were harvested by centrifugation at 8,000 rpm for 20 minutes, in an MSE high speed M-18 centrifuge, washed in distilled water and re-harvested. Organism were lyophilized and stored anhydrously as a fine powder until required and freeze dried for future used.

Extraction of mycolic acids

The procedures adopted in this study by Minnikin., *et al*. [17] involving the acid methanolysis of dried organism followed by TLC (Trace of chromatogram is shown in figure 2 where 100 mg freeze-dried cells were mixed with 5 ml methanol, 5 ml toluene and 0.2 ml concentrated sulphuric acid in 20 ml tubes sealed with PTFE-lined serum caps (How and Cow. Ltd, London.). Methanolysis was carried out

for 16 - 18h at 50°C. The mixture was subsequently cooled and 2 ml hexane was added and the mixture was shaken vigorously. The upper hexane layer was collected. The identity of spots corresponding to mycolic ester was confirmed by washing the developed chromatogram with a mixture of methanol water (3:2 v/v). This procedure remove all spots with RF value less than 0.6 except those corresponding to mycolic esters. The spots with R_f value greater than 0.6 which were nor removed by this washing procedure are chain of fatty acid [18].

•	•	•	•	•	•	•	•	•	•	Fatty acid
		•						•		Mycolic acid
•	•	•	•	•	•	•	•	•	•	
41	C321	JP2/1/2	JP2/1/11	17	JP2/6/3	12	BL8	BL77/18	JP2/6/5	Strain no.

Figure 2: *Tracing of mycolic acid in Thin layer Chromatogram.*

Analysis of mycolic acids

Methyl ester of mycolic acid from hexane extract prepared as described by Minnikin., *et al.* [17] were separated by TLC on I mm thick Silica Gel PF 254-366 plates. These were separated using a slurry (40 g/100 ml) of Merck Silica Gel PF 254-366 in distilled water and dried for 16 - 18h at 65°C. Extract were loaded onto the plates as continuous band and the chromatograms developed with the solvent The samples of hexane extracts spotted on thin layer plates developed in petroleum ether (BP 60 - 80°C): diethyl ether (85:15 v-v). The position of long chain components were revealed by spraying with 30 - 40% aqueous sulphuric acid and charring at 180°C. The components with RF value of 0.1 to 0.5 correspond to methyl ester of mycolic acid or occasionally to methyl ester of 2-hydroxy fatty acids. Components with RF values 0.8 - 1.0 are attributable to the methyl ester of non-hydroxylated fatty acids. The positions of the separated bands were detected under ultraviolet (366 nm) light. Separated components were scraped from the plates and eluted with chloroform into vials and evaporated to dryness under nitrogen.

Results

The presence or absence of mycolic acid was proven to be useful aid in establishing the taxonomic relatedness of some bacteria as coryneform bacteria [18]. Mycolic acid only produced by certain groups of coryneforms which shown to contain both free and bound mycolic acids and they are simpler than those found in *Mycobacterium*, nocardiae and rhodococci [19,20]. The result of 33 strains for the presence or obscener of mycolic acids (Table 1). *Mycobacterium flavum* and the unidentified strains BL 77/18, BL 77/19 and BL77/20 shown to have mycolic acid. The chromatographic mobility of mycolic acid of these strains resembled members of genus *Corynebacterium*. The examination of three stains (C769, C770 and C771) of *Microbacterium liquefaciens* and 6 strains of (C88, C89, C786, C787, C788, C789) of *Microbacterium lacticum* failed to reveal any mycolic acid and therefore differ from *Microbacterium flavum.* These results are not inconsistent with other studies [5,19].

Discussion

The first attempt to correlate lipid composition with taxonomic classification was made by Abdel., *et al*. [21] that showed that qualitative fatty acid analysis could be used to differentiate various organisms. Since then a considerable number of studies were extended and confirmed the usefulness of lipid analysis to microbial taxonomy [21]. In the introduction section the history and results of lipids analysis and contribution to coryneform taxonomy have been reviewed. The results of this study indicated that one part of lipids composition (mycolic acid) of particular value in bacteria taxonomy. The presence of mycolic acids in microbes has proven to be of great value in the chemotaxonomy of coryneform bacteria as described in the introduction. These long chained 3 hydroxy carboxylic acid have been

found in *Mycobacterium, Nocardia*, coryneform and some *rhodococci* where mycolic acids of each group found to be deferent, those of *Mycobacterium* being more diverse in number than those of Corynebacteria [19]. *Corynebacterium diphtheria s*howed to contain both free and bound mycolic acid [5,20], while some other stains were assigned to the genus *Curtobacterium* by other researchers [22-24] because they did not contain mycolic acids. The results obtained in study for the presence or absence of mycolic acid was incorporated in table 1) agreed with studies [5,20] that *Microbacterium lacticum* does not contain mycolic acid and differ from strains of *Microbacterium flavum i*n this respect. This differences support the separation of these two species. *Microbacterium liquefaciens* strains (C769, C770, C771) did not contain mycolic acids a feature not inconsistent with the clustering of theses organism along with *Curtobacterium* as suggested in other studies [22-24]. Four strains out thirty three examined found to have mycolic acid *(M. flavum*, BL77/18, BL77/19, Bl77/20). This results support the relatedness of these strains to each other and to corynebacteria. The failure to detect mycolic acid in strain JP2/1/1 and JP2/1/21 suggested that they may not be similar to *M. flavum* C90. Other strains JP2/1/15 and JP2/1/3 showed no mycolic acid content suggesting no similarity to *M. flavum.* All other unnamed strain tested revealed no mycolic acids and therefore indicate no similarity to *M. flavum* and hence should be place in *Corynebacterium* taxa. The result obtained in this study showed by comparing unspecified isolates to reference strains indicate the important of such acid in detecting close similarity or dissimilarity which might be used to differentiate between taxa. This suggestion is agreed with others studies [1,2]. These results could lay base line for taxonomy of isolates from dairy products and link these isolated to specific taxa that have effect on dairy products deteriorations however future study may required.

Conclusion

The results in this study indicated that *M. lacticum* different from *M. flavum* on the absence of mycolic acid and unknown strain with no mycolic acid is different from *M. flavum* and should be included in *Corynebacterium* taxa. *M. liquefaciens should be placed with Curtobacterium.* The result obtained in this study indicate the important of mycolic acid in detecting similarity or dissimilarity between unspecific strains and reference strains which might be used to differentiate between taxa. This indicate the significant of mycolic acid for taxonomic differentiation.

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