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Short communication

Detection of mycoloylglycerol by thin-layer chromatography as a tool for the rapid inclusion of corynebacteria of clinical origin in the genus *Corynebacterium*

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Abstract

A chemotaxonomic study of some corynebacteria isolated from clinical samples revealed characteristic thin-layer chromatographic patterns for *meso*-diaminopimelic acid containing species included in the genera *Corynebacterium*, *Dermabacter* and *Brevibacterium*. Notably, a specific compound was consistently detected in mycolic acid containing species of the genus *Corynebacterium*. This compound was composed by glycerol and mycolic acids and structural analyses carried out by fast atom bombardment mass spectrometry in *C. minutissimum* confirmed its identification as mycoloylglycerol. The chain length of mycoloyl groups in this molecule ranged from 28 to 34 carbon atoms, being mono-, di- or triunsaturated. Detection of mycoloylglycerol by thin-layer chromatography may be thus useful for the rapid inclusion of a great variety of corynebacteria of clinical origin in the genus *Corynebacterium* in laboratories employing chromatographic techniques as an adjunct for the identification of these microorganisms.

Keywords: *Corynebacterium*; Mycoloylglycerol

1. Introduction

In recent years there has been an increasing interest in corynebacteria due to clinical and taxonomic reasons [1] These microorganisms include the etiological agent of diphtheria and several opportunistic pathogens affecting mainly immunocompromised patients [1]. Applications of genetic approaches in the classification of these bacteria have led to the definition of a great number of new genera and species of clinical relevance [1]. Current methods for identification of corynebacteria include a wide group of biochemical tests, but in most cases this classical approach must be complemented with the analysis of several cell constituents (chemotaxonomy), such as characteristic amino acids and sugars, fatty acids, mycolic acids and phospholipids [2]. Several laboratories have adapted chromatographic techniques, that include TLC [3], GLC [4,5] and HPLC [6,7], as well as GC–MS [8,9] to this purpose.

In this study a variety of corynebacteria containing cell wall *meso*-diaminopimelic acid, obtained in our

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laboratory from clinical samples, were subjected to a chemotaxonomic study by TLC, GLC and GC–MS. When extracts of lipids soluble in chloroform–methanol were examined by TLC, several rather specific patterns were observed for the genera *Corynebacterium*, *Brevibacterium* and *Dermabacter*. Noteworthy was a characteristic compound which was consistently detected in mycolic acid containing *Corynebacterium* species whereas other corynebacteria examined, including *C. amycolatum*, lacked this compound. In the present work, a structural analysis of this compound was carried out and a TLC method is given for its easy detection as a tool for the rapid inclusion of corynebacteria of clinical interest in the genus *Corynebacterium*.

2. Experimental

2.1. Strains analyzed

A total of 103 clinical isolates of corynebacteria were studied (Table 1). For comparative purposes *Corynebacterium amycolatum* NCFB 2768^T, *C. pseudodiphtheriticum* ATCC 10700^T, *C. pseudotuberculosis* ATCC 10950^T and *C. ulcerans* CNCTC 41/74 were also included. Biochemical characteristics of the strains were determined by the API Coryne and the API Zym systems (API Bio-Mérieux, La Balme Les Grottes, France) [11]. For lipids analyses the corynebacteria were cultivated in blood agar [11].

2.2. Lipid analysis

Cells (10–50 mg of wet weight) were extracted with chloroform–methanol (1:1, v/v), overnight at room temperature. The cell pellet and the solvent were separated by centrifugation (1000 *g*, 10 min) and the later evaporated to dryness. The chloroform–methanol lipid extract was then redissolved in 0.2 ml of chloroform–methanol (1:1, v/v) and subjected to TLC analysis on 10×10 cm silica gel TLC plates (cut from 20×20 cm TLC aluminium sheets, silica gel 60 F_{254}, Merck, Darmstadt, Germany), using

Table 1
Strains studied and chemical markers detected; the different lipids were studied by combined TLC, GC and GC–MS; diglycosyldiacylglycerol (DGDAG), MG (mycoloylglycerol) and phospholipids were studied by TLC in chlorofom–methanol (1:1, v/v) extracts; fatty acids were determined by GC and GC–MS, and mycolic acids by TLC and GC–MS

Genus/species (number of strains)	DGDAG	MG	Phospholipids[a]	Mycolic acids (chain length)	Fatty acids (major structural type)
Brevibacterium (16)	+	−	APG, PG, PI	−	*Anteiso*
Dermabacter (3)	+	−	DPG, PG, others[b]	−	*Anteiso*
Corynebacterium amycolatum (24)	−	−	APG, DPG, PG, PI, PIMs	−	Straight chain (TBS)[c]
C. afermentans (10)	−	+	APG, DGP, PG, PI, PIMs	+	Straight chain
C. jeikeium (14)	−	+	APG, DPG, PG, PI, PIMs	+ (28–36)	Straight chain
C. minutissimum (14)	−	+	APG, DPG, PG, PI, PIMs	+ (26–36)	Straight chain
C. propinquum (3)	−	+	APG, DPG, PG, PI, PIMs	+	Straight chain
C. pseudodiphtheriticum (7)	−	+	APG, DPG, PG, PI, PIMs	+ (26–36)	Straight chain
C. striatum (1)	−	+	APG, DPG, PG, PI, PIMs	+	Straight chain
C. urealyticum (2)	−	+	APG, PE, DPG, PG, PI, PIMs	+ (26–34)	Straight chain, TBS
C. xerosis (4)	−	+	APG, DPG, PG, PI, PIMs	+ (26–36)	Straight chain (TBS)[c]
Corynebacterium G2 (3)	−	+	APG, DPG, PG, PI, PIMs	+	Straight chain (TBS)[c]
Corynebacterium F1 (1)	−	+	APG, DPG, PG, PI, PIMs	+ (30–36)	Straight chain (TBS)[c]
Rhodococcus equi (1)	−	+	PE, DPG, PG, PI, PIMs	+	Straight chain, TBS
C. amycolatum NCFB 2768^T	−	−	APG, DPG, PG, PI, PIMs	−	Straight chain
C. pseudiphtheriticum ATCC 10700^T	−	+	APG, DPG, PG, PI, PIMs	+	Straight chain
C. pseudotuberculosis ATCC 10950^T	−	+	APG, DPG, PG, PI, PIMs	+	Straight chain
C. ulcerans CNCTC 41/74	−	+	APG, DPG, PG, PI, PIMs	+ (26–36)	Straight chain

[a] APG, acylphosphatidylglycerol [11]; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositolmannosides.
[b] Include phospholipids migrating like PE, phosphatidylcholine and PIMs.
[c] Some strains contain tuberculostearic acid (TBS) [11].

chloroform–methanol–water (60:12:1, v/v/v) as solvent. The plates were sprayed with molibdophosphoric acid (Merck) (7% w/v, in ethanol) for detection of lipids, and with 0.1% (w/v) 3,5-dihydroxytoluene (orcinol) (Merck)–H_2SO_4 (40%) for sugars, then heated at 120°C until the complete visualization of the separated components. The spot marked as DGDAG (Fig. 1) was purified from *Brevibacterium* sp 25/3. This strain was cultivated in brain heart infusion agar (BHIA) and extracted with chloroform–methanol (1:1, v/v) as above. Purification of DGDAG was performed by preparative silica gel TLC (20×20 cm TLC plastic sheets, silica gel 60, F_{254}) (Merck) employing chloroform–methanol–water (60:12:1, v/v/v) as solvent. Separate components in the plates were revealed with iodine vapor and DGDAG scrapped from the gel, eluted with chloroform–methanol (1:1, v/v) and hydrolyzed at 110°C for 2 h in 1 ml of 2 *M* trifluoroacetic acid. The presence of glycerol (trimethylsilyl ether), sugars (trimethylsilyl ethers) and fatty acids (methyl esters) in this molecule was studied by GC–MS [12]. On the other hand, spot marked as CF (cord factor) (Fig. 1), was purified as DGDAG in the strain *C. afermentans* 13524, and the presence of trehalose (trimethylsilyl ethers) and mycolic acids (methyl esters) determined by GC–MS [12] after hydrolysis at 110°C for 2 h in 1 ml of 2 *M* trifluoroacetic acid.

Phospholipids were determined by TLC as previously described [11].

The fatty acid content of all strains was studied by GLC [12] in the chloroform–methanol extract after acid methanolysis [3], and their structures confirmed by GC–MS [12]. Mycolic acids of several representative strains (see Table 1) were studied from the cell pellet, liberated by acid methanolysis [3], purified by TLC, transformed to trifluoroacetyl derivatives [12] and analyzed by GLC and GC–MS [12].

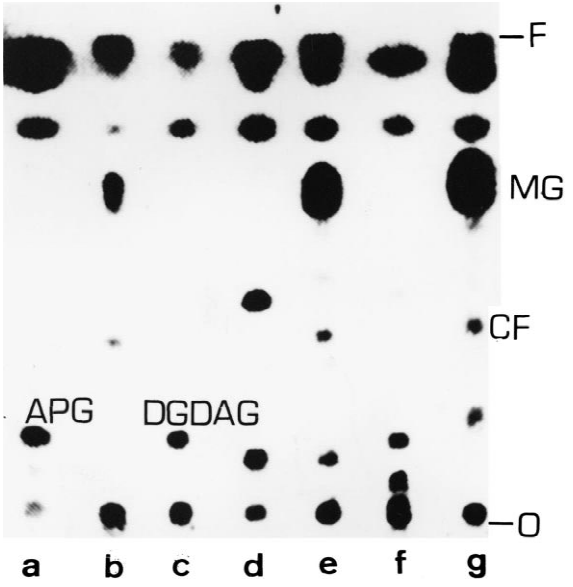

Fig. 1. TLC of soluble lipids in chloroform–methanol (1:1, v/v) of several corynebacteria: lanes: (a) *Corynebacterium amycolatum*; (b) *C. minutissimum*; (c) *Brevibacterium* sp; (d) *Dermabacter* sp; (e) *C. jeikeium*; (f) *Dermabacter* sp; (g) *C. minutissimum*. Solvent: chloroform–methanol–water (60:12:1, v/v/v). The plates were revealed at 120°C/10 min, after spraying with molibdophosphoric acid (7%, w/v, in ethanol). MG, mycoloylglycerol; CF, cord factor; DGDAG, diglycosyldiacylglycerol; APG, acylphosphatidylglycerol. O, origin; F, solvent front. Lipids near F are mixtures of acylglycerols and cholesterol (the latter being incorporated from the blood agar by the cells).

2.3. Structural analysis of mycoloylglycerol

The compound designated MG (mycoloylglycerol) (Fig. 1) was purified from *Corynebacterium minutissimun* 3578/2, that was cultivated in BHIA. The cells were extracted with chloroform–methanol (1:1, v/v) and the lipids examined by TLC using chloroform–methanol–water (60:12:1, v/v/v) as solvent. MG was purified by preparative TLC as described for DGDAG, and subjected (1 mg) to acid methanolysis [3]. The presence of glycerol (trimethylsilyl ether) was studied by GC–MS [12]. Liberated mycolic acid methyl esters from MG were detected by TLC [3], transformed to trifluoroacetyl derivatives and studied by GC–MS [12]. Fast atom bombardment (FAB) MS of MG (1 mg) was performed in a VG AutoSpec mass spectrometer, with *m*-nitrobenzyl alcohol as matrix.

2.4. Other chemotaxonomic markers

meso-Diaminopimelic acid was studied by TLC and whole cell sugars (trimethylsilyl ethers) by GLC [11,12].

3. Results and discussion

3.1. Chemotaxonomic markers of the strains studied

All the strains analyzed contained *meso*-diaminopimelic acid but, as expected [3,13,14], differed in the fatty acid patterns, phospholipids constituents and content of mycolic acids (Table 1). Characteristic sugars of members of the genus *Corynebacterium* were identified as arabinose, galactose and mannose [2]. In the genus *Brevibacterium* the sugar pattern was more complex, with neither arabinose nor galactose present, but with glucose, ribose, galactosamine and glucosamine as characteristic compounds, accompanied by ribitol, a composition previously reported for some brevibacteria, and probably corresponding to their content in teicoic acids [15]. No studies on whole-cell sugar composition of *Dermabacter* strains were carried out.

3.2. Mycoloylglycerol and other compounds

When lipids soluble in chloroform–methanol (1:1, v/v) were examined by TLC employing chloroform–methanol–water (60:12:1, v/v/v) as solvent, several characteristic components were detected (Table 1; Fig. 1), which were designated MG, CF, diglycosyldiacylglycerol (DGDAG) and acylphosphatidylglycerol (APG).

The presence of DGDAG in *Brevibacterium* was suspected after positive detection with molibdophosphoric acid or orcinol of a spot with an R_f value of 0.17–0.20 (Fig. 1c). After purification and acid hydrolysis of this compound in the strain *Brevibacterium* sp 25/3, glycerol, mannose and fatty acids (mostly — 75% — *anteiso*-pentadeconoic and *anteiso*-heptadecanoic) were detected. Hence, the identification of this compound as dimannosyldiacylglycerol was proposed. The DGDAG content in members of the genus *Brevibacterium* has been reported previously [15]. Glycolipids with chromatographic mobility similar to DGDAG revealed in *Dermabacter* strains (Fig. 1d and f) were not characterized further; however, related compounds have been presumptively identified as DGDAG in *Dermabacter hominis* [13]. In most species of the genus *Corynebacterum* a compound designated APG was present in minor amount, although it was a major spot in the TLC profiles of *C. amycolatum* (Fig. 1, lane a) [11]. APG migrated like DGDAG, but, unlike this compound, it was not revealed with orcinol–sulphuric acid reagent. This property can be used to distinguish *C. amycolatum* from *Dermabacter* and *Brevibacterium*, which is an important feature because *C. amycolatum* is one of the non-lipophilic corynebacteria more frequently isolated in clinical laboratories [1].

The TLC system cited above also revealed a component designated MG (R_f 0.6–0.7; Fig. 1b,e,g) in all the species of *Corynebacterium* with mycolic acids (Table 1). This compound was, in most cases, accompanied by cord factor (CF). This last compound liberated trehalose and mycolic acids after hydrolysis, and was consequently identified as CF (6,6′-dimycoloyl trehalose) (R_f 0.40–0.45; Fig. 1b,e,g), a typical constituent of a variety of bacteria containing mycolic acids [16]. Several strains of *C. minutissimum*, *C. urealyticum* and *C. xerosis* were devoid of CF. When CF and MG were present together, MG concentration was, in general, higher than CF, according to the size of the spots, although *C. afermentans* and some strains of *C. jeikeium* apparently contained higher amounts of CF.

Mycoloylglycerol purified from *Corynebacterium minutissimum* 3578/2 liberated glycerol and mycolic acids. Glycerol (as trimethylsilyl ether) was positively identified by GC–MS, because of the presence in the mass spectrum of several fragments at *m/z* 73, *m/z* 103, *m/z* 117, *m/z* 133, *m/z* 147, *m/z* 205, *m/z* 218 and *m/z* 293 (M^+-15). The chain length of mycolic acids liberated from MG varied from 28 to 34 carbon atoms, being identified by GC–MS as (carbon atoms and double bonds): 28.1, 30.2, 30.1, 32.3, 32.2 and 34.3. The FAB mass spectrum of MG (not shown) was dominated by a series of peaks at *m/z* 539 (intensity 53.8%), *m/z* 541 (82.2%), *m/z* 565 (44%), *m/z* 567 (100%) and *m/z* 593 (37.4%), corresponding to $M^+ + H^+$ ions of MG with mycoloyl groups identified, respectively, as 30.2, 30.1, 32.3, 32.2 and 34.3. Minor $M^+ + H^+$ ions for MG with mycoloyl groups of 28.1 (*m/z* 513, intensity 8.3%) and 30.3 (*m/z* 537, intensity 12.8%)) were also present in the FAB mass spectrum.

The presence of MG, identified as 1-mono-

mycoloylglycerol, has been previously detected in a limited number of strains of the genera *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Corynebacterium* [10,17,18], but no systematic analyses for this compound have been reported in these microorganisms. The chain length of mycoloyl groups present in MG is similar to those found attached to the cell wall in each of the cited genera. Thus, in *Corynebacterium pseudotuberculosis*, the only species of the genus so far studied, it varied from 30 to 36 carbon atoms, with a major component of 32 carbon atoms [10].

Our data suggest that MG is distributed in all the species of the genus *Corynebacterium* with the only exception of *C. amycolatum*, a species lacking mycolic acids [19], although the recently described species *C. kroppenstedtii* [20] is also devoid of mycolic acids. Thus, detection of MG by TLC is an useful tool for the rapid inclusion of a great variety of corynebacteria of clinical origin in the genus *Corynebacterium*. Mycoloylglycerol has a characteristic R_f value, and, according to our results and those found in the literature [2,15,21], no other lipids detected in either the corynebacteria studied or related microorganisms show a similar mobility on TLC. This technique avoids misidentification of coryneform bacteria with true members of the genus *Corynebacterium* in a single chromatographic step, without analyses of fatty and mycolic acids or other cell chemical markers. It should be noted, however, that *Rhodococcus equi*, a species implicated in pulmonary infections in immunocompromised (AIDS) patients and frequently isolated in clinical laboratories, also produces MG with similar chromatographic behaviour to that found for *Corynebacterium* (Table 1). Nevertheless, *Rhodococcus equi* and the remaining species of this genus, as well as *Nocardia* and related bacteria producing this kind of molecules [17,18], differ from *Corynebacterium* by morphological features.

In conclusion, mycoloylglycerol, a compound widely distributed in *Corynebacterium* species containing mycolic acids, can be easily detected by TLC, and this technique appears to be useful for the rapid inclusion of corynebacteria of clinical interest in the genus *Corynebacterium*.

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