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Short communication

Pyrolysis–gas–liquid chromatography with atomic emission detection for the identification of *Corynebacterium* species

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Abstract

We report here the application of pyrolysis–gas chromatography followed by atomic emission detection (AED) for the characterisation of microorganisms. AED measured the quantity of carbon, sulfur and nitrogen in the molecules separated chromatographically. Twenty-three strains, representing eight *Corynebacterium* species, were tested in this preliminary study. Co-ordinate principal analysis grouped 11 strains in their respective species group. Most of the other strains appear randomly distributed, perhaps because these strains require additional nutrients. These preliminary results show that the method could be used as a tool for the taxonomic and perhaps the epidemiologic characterisation of bacteria.

Keywords: *Corynebacterium* spp.; Bacteria; Pyrolysis; Chemometrics

1. Introduction

Pyrolysis is a chemotaxonomic method used since 1965 for species level identification and classification of micro-organisms [1]. The substrate is rapidly heated until it reaches a pre-set temperature, in an inert atmosphere (helium, nitrogen) or in vacuum [2]. This temperature is usually between 300 and 1200°C. Gaseous molecules escape from the substrate, and form a characteristic chromatographic pattern, called a pyrogram.

Connected to gas chromatography–flame ionisation detection (GC–FID) or to mass spectrometry (MS), pyrolysis (Py) is a effective method for chemotaxonomy [3–5]. Py–MS is more difficult to use, but is faster and more sensitive. Other chemotaxonomic methods exist, like fatty acid profiling or DNA analysis. Many of them are automated and are much more effective than pyrolysis techniques.

However, we think that pyrolysis techniques can resolve classification problems for poorly known bacteria from the genus *Corynebacterium*. In this preliminary study, we just want to lay the basis for a

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novel approach by pyrolysis and gas chromatography.

We have replaced FID with atomic emission detection (AED). This detection method has never been used to characterise microorganisms. AED selectively detects some atomic elements in the molecules eluted from GC, thanks to a microwave-induced helium plasma [6,7]. The high ionisation energy of helium atomise the molecules and excite the atoms, which emit their specific emission rays [8]. These photons are collected by a photodiode array as a function of time [9]. Reagent gases (oxygen, hydrogen) are added to improve sensitivity and prevent carbon deposition on the wall of discharge tube. AED selectively detect 29 elements, especially atoms of organic chemistry. During a single run, up to four elements are detected.

The 23 studied strains belong to the genus *Corynebacterium*. These are commensal bacteria, frequently involved in nosocomial infections and sometimes difficult to identify [10,11]. New characterisation methods may be of interest to microbial taxonomy and epidemiology.

We used a small number of strains, but these strains represent many of the most common species from the genus *Corynebacterium*. Future studies will use more strains in each species and will give more information about classification of the genus *Corynebacterium*.

2. Materials and methods

2.1. Gases

Helium quality 5.5 (Air Products, Paris, France) was used as carrier gas for pyrolysis and GC. AED plasma was generated with this gas. The reagent gases used here were oxygen quality 4.5 and hydrogen quality 5. Argon quality U was used to purge the optical part of the AED system.

2.2. Bacterial strains

We studied 23 strains representing eight *Corynebacterium* species (see Table 1). Seven strains came from international collections, the others were clinical strains, which were not epidemiologically related.

Strains were recovered from our stock collection and grown on Trypcase-Soja agar with 5% sheep blood (BioMerieux, Marcy l'Etoile, France), during 48 h at 37°C and under a CO_2-rich atmosphere. Then they were isolated under the same cultural conditions. A 100-ml volume of Brain-Hearth liquid medium was inoculated with a single colony. After 48 h at 37°C with slow shaking, a purity control was made on an agar plate, then the bacteria were killed by adding 2.5 ml of formaldehyde. Half an hour later, in order to verify inactivation, a drop of bacterial suspension was dropped onto an agar plate. Then the bacteria were recovered by centrifugation (10 000 *g*, 20 min, 4°C). The pellets were washed twice in distilled water, then freeze dried.

2.3. Pyrolyser

We used a Pyrojector II (SGE, Victoria, Australia). It is a furnace pyrolyser: a cylindrical heating zone surrounds the vertically quartz liner, which receives the samples. The selected temperature was 500°C, the internal helium pressure was 13.6 p.s.i. (94.8 kPa).

For each strain, 100 μg of bacteria suspended in water (Millipore Milli-Q quality) was introduced into a glass capillary tube, which had an internal volume of 1 μl. Water was removed by drying at 60°C, then this tube was manually placed in the pyrolyser quartz liner by means of a syringe. Inside the liner, a glass wool plug held the sample. Triplicate assays were performed. The quartz liner was replaced after two pyrolysed strains. A blank run was performed before each series.

2.4. Chromatographic conditions

A HP 6890 GC system (Hewlett-Packard, Palo Alto, CA, USA) with split/splitless inlet was used. We used a capillary column from Hewlett-Packard: the 19091J-413 HP-5, with 5% phenyl-methylsiloxane, 30 m×320 μm, 0.25 μm film thickness.

The inlet was used in splitless mode, at a constant pressure of 8.6 p.s.i. (59.3 kPa), with purge at 40 ml/min after 2 min and flow reduced at 20 ml/min

Table 1
Bacterial strains

Experiment No.	Species	Collection strain[a]	Clinical strain[b]
1	*C. striatum*		5996393
2	*C. striatum*	NCTC 764	
3	*C. striatum*	CDC 5333	
4	*C. urealyticum*		19495331
5	*C. urealyticum*		19495332
6	*C. urealyticum*		19495333
7	*C. pseudotuberculosis*	CDC F3477	
8	*C. jeikeium*	CIP 103337 T	
9	*C. jeikeium*		33495360
10	*C. jeikeium*		4396372
11	*C. amycolatum*		1839752
12	*C. amycolatum*	CIP 103452 T	
13	*C. amycolatum*		18397473
14	*C. amycolatum*		18397474
15	*C. diphtheriae*		9695147
16	*C. diphtheriae*		9695149
17	*C. pseudodiphtheriticum*		8795134
18	*C. pseudodiphtheriticum*		8795132
19	*C. pseudodiphtheriticum*		8795136
20	*C. minutissimum*	NCTC 10288[c]	
21	*C. minutissimum*		5996395
22	*C. minutissimum*		18397475
23	*C. minutissimum*	ATCC 23348[c]	

[a] ATCC: American Type Culture Collection, Manassas, VA, USA. CDC: Center Disease Control, Atlanta, GA, USA. CIP: Collection of Institute Pasteur, Paris, France. NCTC: National Collection of Type Culture, London, UK. T: Type strain.
[b] Collection of F.N.R. Renaud and D. Aubel, IUT A, UCBL, Villeurbanne, France.
[c] Strains designed as synonym.

30 s later. The inlet liner was a single tapered HP 5581-3316. The inlet temperature was 220°C.

The oven temperature was held at 65°C during 4 min, then increased at 15°C/min to 250°C, then held for 5 min. The GC–AED transfer line was heated at 270°C.

The AED system was the HP 2350 A Model. The following elements were simultaneously detected: carbon (wavelength 193.03 nm), sulfur (180.7 nm) and nitrogen (174.299 nm). The plasmagene gas flow was set at 50 ml/min. The reagent gas pressures were 23.65 p.s.i. (192.7 kPa) for oxygen and 9.78 p.s.i. (67.4 kPa) for hydrogen.

2.5. Analysis

The software HP Chemstation A.03.04, which controlled the GC–AED system, calculated the areas of the peaks for the three wavelengths. These areas were verified, then the values for retention times (t_R) and areas for all strains were collected in a spreadsheet file for treatment before statistical comparison.

The peaks were identified thanks to their t_R values. Retention times could have a variation of 3 s, because samples were manually introduced. For each element pattern from each assay, the peak, which had the most important area, was searched. This peak received the factor 100, then all others areas received a calculated factor in proportion with this highest area. These factors were used for comparison of patterns. Only the most reproducible peaks are considered in statistical analysis. The peaks with an area less than 10% in comparison with the highest peak were not used. In the same way, we did not use

the peaks which had a relative standard deviation (RSD) higher than 25% for the three replicate analysis of the same strain. All these procedures are automated.

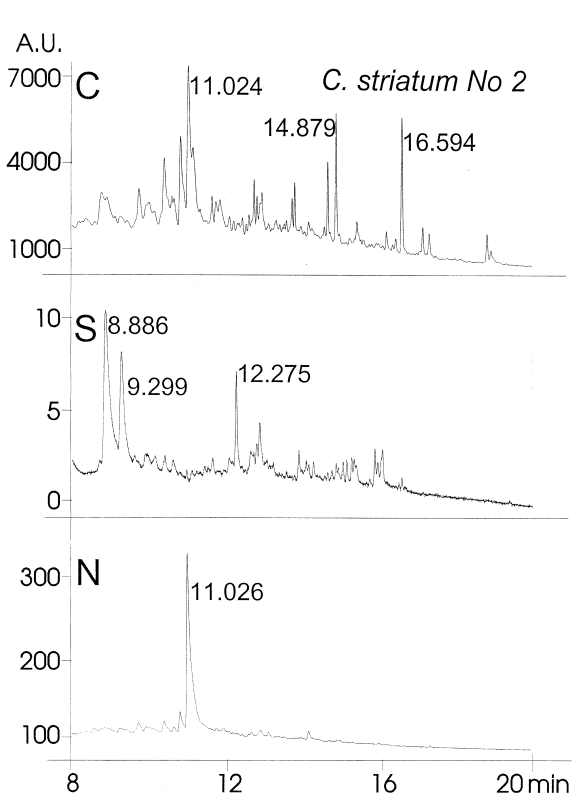
For the remaining areas, the average peaks were calculated for the three replicates. They were compared by the software Taxotron (Professor P.A.D. Grimont, Taxolab, Institut Pasteur, Paris, France). The program Taxotron calculated Euclidean distances based on the data series. With the distance matrix, a subprogram carried out a co-ordinate principal analysis (CPA). With these results, a graph was drawn, showing the strain dispersion.

3. Results

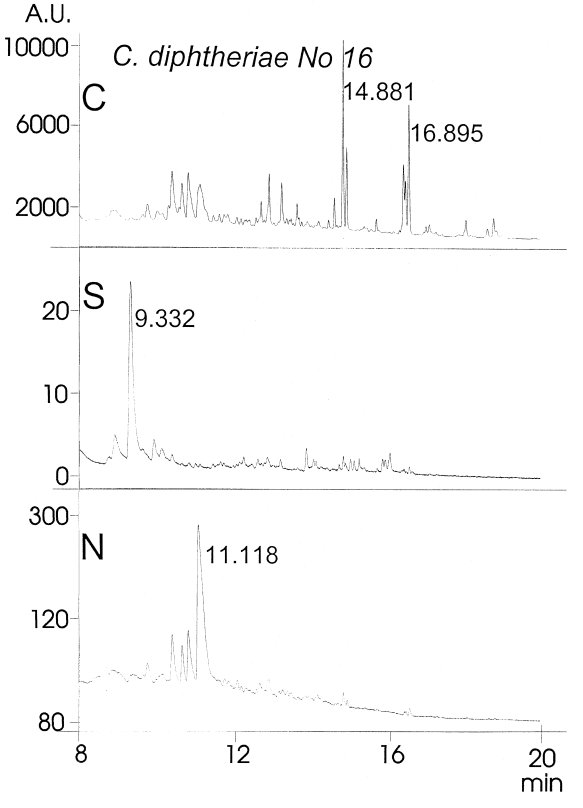
Typical chromatograms for each of the three wavelengths are shown in Fig. 1. There are a few changes between patterns of different strains. But these differences do not allow an objective classification. The data treatment selected 65 peaks (39 from the carbon patterns, 11 from sulfur, 15 from nitrogen) over 118. The CPA provided results shown in Fig. 2.

The statistical analysis grouped 11 strains by species. The three strains (Nos. 1, 2 and 3) of *C. striatum* are grouped together. In the same way, three strains of *C. amycolatum* (Nos. 12, 13 and 14), three strains of *C. minutissimum* (Nos. 20, 21 and 22), and two strains of *C. pseudodiphtheriticum* (Nos. 18 and 19) are grouped by species. But the *C. amycolatum* and the *C. minutissimum* groups are mixed.

Nine strains are placed far from their respective group. At first, *C. minutissimum* (No. 23), *C.*



(a)



(b)

Fig. 1. Pyrolysis patterns of *C. striatum* No. 2 (a) and *C. diphtheriae* No. 16 (b). C: carbon (193.03 nm), S: sulfur (180.7 nm), N: nitrogen (174.299 nm). AU: Arbitrary units for photodiode detection.

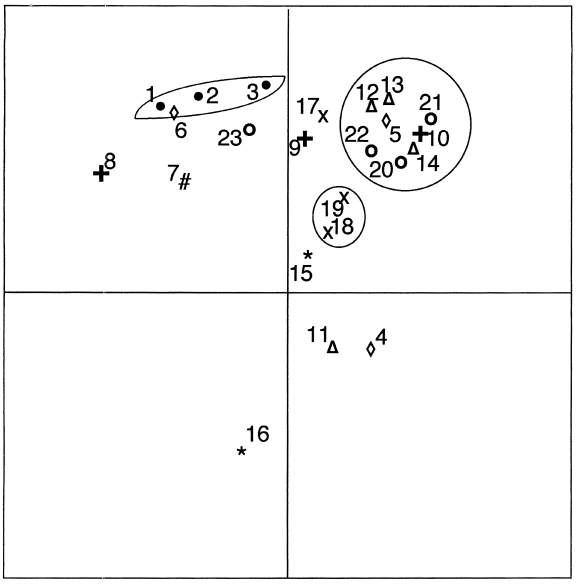


Fig. 2. Co-ordinate principal analysis from the pyrolysis patterns of 23 *Corynebacterium* strains. 1, 2 and 3: *C. striatum*; 4, 5 and 6: *C. urealyticum*; 7: *C. pseudotuberculosis*; 8, 9 and 10: *C. jeikeium*; 11, 12, 13 and 14: *C. amycolatum*; 15 and 16: *C. diphtheriae*; 17, 18 and 19: *C. pseudodiphtheriticum*; 20, 21, 22 and 23: *C. minutissimum.*

amycolatum (No. 11), and *C. pseudodiphtheriticum* (No. 17) are not in the precedent groups. On the other hand, the three strains of *C. jeikeium* and the three strains of *C. urealyticum* are all randomly distributed. These dispersions reduce the significance of results.

The unique strain of *C. pseudotuberculosis* (No. 7) is aside other strains. The two *C. diphtheriae* strains (Nos. 15 and 16) are also aside. But these last two strains are far from one another: the two strains of *C. diphtheriae* are not regrouped.

4. Discussion–conclusion

The use of pyrolysis in analytical microbiology, with GC–FID and with MS, was frequently compared with biochemical tests, DNA hybridisation or another conventional method. These studies show that pyrolysis is able to classification and identification of bacterial strain populations at species or subspecies level in the same way as reference methods. For example, pyrolysis and MS confirmed conventional tests in classification of *Corynebacterium* strains belonging to many species [12]. A classification congruence of the strain classification for other micro-organisms was showed [13–15]. The aim in these studies is a species or a biotope separation, and the pyrolysis fingerprints provided the same classification as results obtained by chemical, biochemical or genetic methods.

At the epidemiological level, results may vary. A *Haemophilus influenzae* population study showed different results depending on the used method: Py–MS, ribotyping, protein pattern [16]. However, for other species, the pyrolysis provides an exact separation in subspecies groups: the accuracy of these methods can confirm a nosocomial outbreak or show an unexpected diversity in a culture [17–19]. The epidemiological qualities of pyrolysis are proved and concern bacteria and yeast like *Candida albicans* [20].

Py–MS is faster and more sensitive than Py–GC–FID, but a GC system has more applications in a reference microbiology laboratory. We attempted here to set a simple and objective characterisation method of *Corynebacterium* species. One of the drawbacks of Py–GC–FID is the poor reproducibility and large background. Here, we replaced FID with a multi-element selective detection method, AED, and we used a HP-5, a non-polar chromatographic column, steadier than the Carbowax 20M, the column used in the most studies on Py–GC–FID. With these changes, we hope to increase efficiency.

With the method reported here, the *C. amycolatum* and the *C. minutissimum* groups are indistinct, which is in accordance with their phenotypic proximity. These two species are very close, and are hard to separate by usual phenotypic methods [11].

On the other hand, pyrolysis show specific markers of *C. striatum*: the three *C. striatum* strains are clearly separated from strains of *C. amycolatum* and *C. minutissimum*, although *C. striatum* is phenotypically close to these two species.

The *C. jeikeium* and *C. urealyticum* strains, which are randomly distributed in Fig. 2, belong to lipophilic species. They easier grow on a medium added with fatty acids. In order to respect an essential condition in chemotaxonomy, we used the same cultural conditions for each strain, lipophilic or not lipophilic: we used a rich medium, ordinary blood agar. A lipophilic strain can slowly grown on blood

agar. But the lack of fatty acid may induce a divergence between strains. Indeed, the pyrolysis results are dependent of the bacterial culture conditions [21]. In the next study, we will compare the use of blood agar and blood agar added with fatty acid.

In the case of *C. minutissimum*, the reference strain number 23 is separated from its synonym, the reference strain 20. All strains were re-identified after culture by API Coryne system (BioMerieux), and the strains 20 and 23 were confirmed to belong to *C. minutissimum* species. We cannot explain this separation: perhaps a mutation occurred in one of the two strains.

As with Py–GC–FID, Py–GC–AED could characterise bacterial strain populations at species level and perhaps at subspecies level. However, this study must be continued on a greater number of strains and species, in order to specify pyrolysis markers, similarities with others phenotypic methods and limits of reproducibility.

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