Cellular Fatty Acids as Chemical Markers for Differentiation of *Acinetobacter baumannii* and *Acinetobacter calcoaceticus*  

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Abstract

**Objective** Gas chromatography (GC) was used to investigate the cellular fatty acid (CFA) composition of 141 *Acinetobacter baumannii* and 32 *A. calcoaceticus* isolates from different locations in China and to find chemical markers to differentiate these two closely related bacteria.

**Methods** Whole cell fatty acid methyl esters (FAMEs) were obtained by saponification, methylation, and extraction for GC analysis, followed by a standardized Microbial Identification System (MIS) analysis.

**Results** All *A. baumannii* and *A. calcoaceticus* strains contained some major fatty acids, namely, 18:1 ω9c, 16:0, Sum In Feature 3, 12:0, 17:1ω8c, 3-0H-12:0, 17:0, Sum In Feature 2, 2-0H-12:0, and 18:0 compounds. Although most of the total CFAs are similar between *A. baumannii* and *A. calcoaceticus* strains, the ratios of two pairs of CFAs, i.e., Sum In Feature 3/18:1 ω9c versus 16:0/18:1 ω9c and Sum In Feature 3/18:1 ω9c versus unknown 12.484/18:1 ω9c fatty acids, could differentiate these two closely related bacteria. *A. baumannii* could be easily classified into two subgroups by plotting some ratios such as Sum In Feature 3/16:0 versus 17:0 and Sum In Feature 3/2-0H-12:0 versus17:0 fatty acids.

**Conclusion** The ratios of some CFAs could be used as chemical markers to distinguish *A. baumannii* from *A. calcoaceticus*.

**Key words:** *Acinetobacter baumannii*; *Acinetobacter calcoaceticus*; Differentiation; Fatty acids; Gas chromatography.

INTRODUCTION

*Acinetobacter* bacteria are gram-negative, strictly aerobic, non-motile coccobacilli that are commonly distributed in soil and water of natural environments[1]. Some of these bacteria are also important nosocomial pathogens. These bacteria are usually associated with skin colonization of hospitalized patients and have been associated with serious infections. The genus *Acinetobacter* currently contains up to 23 described and named, as well as several unnamed (genomic), species[2]. Three of the most clinically relevant species, *A. baumannii*, genomic species 3, and 13TU, are

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*This research was supported by the Innovation Foundation of Shanxi Medical University for undergraduate students (No. 2009056) and the National Key Program for Infectious Diseases of China (No. 2008ZX10004-009).

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Received: December 12, 2011; Accepted: June 5, 2012
commonly grouped with the environmental organism *Acinetobacter calcoaceticus* in the *A. calcoaceticus–A. baumannii* complex because of the difficulty in differentiating these species using phenotypic methods[3-6].

In recent decades, *A. baumannii* has increasingly been implicated in outbreaks of nosocomial infections causing pneumonia, bacteremia, urinary tract infections, wound infections, and meningitis worldwide because of its innate and acquired antimicrobial resistance as well tendency for epidemic spread[5-6]. The increasing rates of the resistance of this species to the major antimicrobial agents necessitate early identification and control of nosocomial outbreaks. There are several successful epidemic *A. baumannii* strains (clones) circulating in Europe according to recent data. Thus, a better understanding of the diversity within the species and the emergence of epidemic clones is needed[7-9].

Several methods are used to identify the *Acinetobacter* species, including phenotypic features and genetic markers. DNA-DNA hybridization is the standard method to differentiate these species, but this technique is very laborious, which prevents its routine application in clinical laboratories. The phenotypic identification scheme proposed by Bouvet and Grimont in 1986 was based on 28 phenotypes[10]. However, this scheme could not distinguish the closely related and clinically relevant *A. calcoaceticus–A. baumannii* complex[3]. Many molecular methods have been developed and validated for the identification of acinetobacters, including the amplified 16S rRNA gene restriction analysis (ARDRA)[11], the high-resolution fingerprint analysis by amplified fragment length polymorphism (AFLP)[12-13], ribotyping[14], tRNA spacer fingerprinting[15], and so on. ARDRA and AFLP analyses are currently the most widely accepted methods for species identification of acinetobacters, with a large library of profiles available for both reference and clinical strains. However, these techniques are time-consuming. Recently, new methods have been proposed for the identification of *Acinetobacter* isolates to the species level mainly based on polymerase chain reaction (PCR) and sequencing technologies. These techniques include sequencing of the *rpoB* gene and its flanking spacer regions[16] (as well as the 16S-23S rRNA gene spacer region[17]), PCR-based method that exploits differences in *gyrB* gene sequences[17], detection of the intrinsic blaOXA-51-like carbapenemase gene[18], and genotyping by multilocus PCR as well as mass spectrometry[19]. These methods, based on their detection of nucleic acid, have contributed to a better understanding of the epidemiology and clinical significance of *Acinetobacter* species in recent years. However, new methods should be developed to provide alternative choices for professionals.

Aside from the methods described above, the determination of cellular fatty acid (CFA) composition by gas chromatography (GC) may serve as an alternative method to distinguish the *Acinetobacter* species. As early as 1963, Abel et al.[20] and Kaneda presented evidence that suggested the probable successful use of CFA for bacterial identification[21-22]. Other early studies facilitated the establishment of CFA analysis as a widely accepted method for bacterial identification and classification[23]. The normalization of the bacterial fatty acid analysis method with GC is required for parallel comparison of CFA results from different laboratories. The improvement of the Sherlock Microbial Identification System (MIS) and Sherlock standard libraries (MIDI Corporation) has made realization of this goal possible. The purpose of the present study is to analyze the fatty acid composition of different strains of *A. baumannii* and *A. calcoaceticus* using the Sherlock MIS and to determine the differentiation capability of fatty acid patterns.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Condition**

**Bacterial Strains** A total of 141 strains of *A. baumannii* and 32 strains of *A. calcoaceticus* were collected from eight provinces in China. All these strains, isolated between 2008 and 2009 from 10 different hospitals in different cities in China, were identified by conventional biochemical features and confirmed at the species level by ARDRA[11]. The sources of the strains are provided in the supplementary Table S1.

**Growth Conditions** Fatty acids were extracted and analyzed by following the Sherlock MIS protocol (MIS, MIDI Inc., Newark, DE)[24]. The growth medium was trypticase soy broth agar (BD, USA). The cultures were incubated for 24 h at 28 °C to allow the population to reach the stationary growth phase where the fatty acid composition is rather stable.

**Chemical Procedures and GC**

CFAs were extracted and transformed into fatty acid methyl esters (FAMEs) by using the method recommended by the Sherlock MIS protocol. The
following procedures were performed: (i) saponification at 100 °C for 30 min after adding 1 mL of 15% (w/v) NaOH in 50% methanol; (ii) methylation of the released fatty acids at 80 °C with 2 mL of methanolic HCl (325 mL 6 N HCl mixed with 275 mL methanol) for 10 min; (iii) extraction of the FAMEs into 1.25 mL of 1:1 (v/v) ether and hexane; and (iv) washing of the organic extract with 3 mL of 1.2% (w/v) NaOH.

FAMEs were analyzed by GC by following the MIS operation manual[24]. The gas chromatographic unit consists of a Hewlett-Packard 6890 module (Version A.03.02) equipped with a 25 m × 0.2 mm cross-linked 5% phenylmethyl silicone fused-silica capillary column (Ultra-2, HP 19091B-102, Hewlett-Packard Co.), a flame ionization detector, and a 7673 automatic sampler (Hewlett-Packard Co.). The column temperature ramps from 170 °C to 260 °C at the speed of 5 °C per min, then increases to 310 °C at the speed of 40 °C per min, and finally is kept at 310 °C for 1 min. Hydrogen serves as the carrier gas at the addition speed of 0.5 mL per min. The FAMEs were identified and qualified by the Sherlock MIS software (ver. 4.5) according to their equivalent chain value.

**Statistical Analysis**

The relative quantitative data of CFA profiles obtained by the Sherlock MIS are provided in supplementary Table S1. The means of the CFA contents of different bacteria from different locations were analyzed by ANOVA of SAS system (Version 8.2, SAS Institute Inc., Cary, NC, USA). Statistical significance was accepted when P<0.05.

**RESULTS**

**Fatty Acid Composition of A. baumannii**

The average CFA percentage of A. baumannii strains obtained from different locations is presented in Figure 1a. All strains possess CFAs with almost identical characteristics. The most significant CFA is 18:1 ω9c, with an average proportion of approximately 42%. The other two major CFAs are 16:0 (19.2%) and Sum In Feature 3 (comprising iso-2-OH-15:0 and/or 16:1 ω7c; 13.6%). These three CFAs account for approximately 70% of the total CFAs, similar to those reported previously[25-27]. The proportions of 12:0, 17:0 ω8c, 3-OH-12:0, 17:0, Sum In Feature 2 (comprising 16:1 ISO I and/or 3-OH-14:0), 2-OH-12:0, and 18:0 are relatively lower.

In the present study, the minor fatty acids detected in the different strains of A. baumannii are 10:0, unknown 12.484, 13:0, Sum In Feature 1 (comprising 15:1 ISO H and/or 3-OH-13:0), 15:1 ANTEISO A, ISO 3-OH-12:0, 15:1 ISO F, and 16:1 ω9c, with the average contents of less than 1%.

**Fatty acid Composition of A. calcoaceticus**

The CFA profiles of A. calcoaceticus isolates are shown in Figure 1b. The proportion of 18:1 ω9c fatty acid (more than 28%) is the highest among the CFAs in this bacterium. The Sum In Feature 3 and 16:0 are the second and the third most abundant, with an average proportion of 24% and 20%, respectively. Other components such as 12:0, 17:1ω8c, 3-OH-12:0, 18:1 ω7c, 15:0, Sum In Feature 2, 18:0, and so on, are similar to those in A. baumannii strains.

**Comparison of CFAs between A. baumannii and A. calcoaceticus**

The differences in fatty acids among A. baumannii and A. calcoaceticus strains were assessed by ANOVA. P values were both less than 0.01 in 18:1 ω9c and Sum In Feature 3 fatty acids. The P values in 18:1 ω7c, 18:0, 17:0, and 14:0 were less than 0.05 between the two different species (Figure 2).

**A. baumannii Subgrouping by Fatty Acid Markers**

Using the standardized procedure for analyzing fatty acids, A. baumannii could be easily classified into two subgroups by plotting some ratios such as Sum In Feature 3/16:0 versus 17:0 and Sum In Feature 3/2-OH-12:0 versus17:0 fatty acids (Figure 3).

**Chemical Markers for Differentiating A. baumannii and A. calcoaceticus**

By plotting the ratios of the Sum In Feature 3/18:1 ω9c versus 16:0/18:1 ω9c and unknown 12.484/18:1 ω9c versus Sum In Feature 3/18:1 ω9c fatty acids (Figure 4), two clusters were clearly observed for each of the two species.

**DISCUSSION**

The reproducibility of the CFA analysis by MIS was confirmed previously in the authors’ laboratory, indicating that the major CFA compositions (those above 1% of the total fatty acids) of the samples are stable and that the variance coefficients of different fatty acids vary from 0.52% to 6.69%[28].
Figure 1. a, Histogram based on the CFAs of all A. baumannii strains. b, Histogram based on the CFAs of all A. calcoaceticus strains.

Figure 2. Histogram based on the CFAs of all strains studied. Fatty acids marked with "*" stand for the significant difference between the two species.
Comparison of CFAs of \textit{A. baumannii} and \textit{A. calcoaceticus} strains from Different Locations

The differences in fatty acids among the strains from different locations were assessed by ANOVA. The results demonstrate that the $P$ values were greater than 0.05 in all fatty acids, indicating a significant similarity in CFA profiles of different strains from different locations. In general, the fatty acid compositions of \textit{A. baumannii} (Figure 1a) or \textit{A. calcoaceticus} (Figure 1b) strains are very conservative such that discriminating their sources by CFA profiles is impossible.

Comparison of CFAs between \textit{A. baumannii} and \textit{A. calcoaceticus}

From Figure 2, 18:1 $\omega$9c and Sum In Feature 3 fatty acids represent a significant distinction between \textit{A. baumannii} and \textit{A. calcoaceticus}. Aside from these two acids, there are other fatty acids whose contents have distinct differences between the two species, such as 18:1 $\omega$7c, 18:0, 17:0, 14:0, and so on. However, the contents of five fatty acids (12:0, 15:0, 3-OH-12:0, 2-OH-12:0, and Sum In Feature 2) were found to be highly similar. Notably, approximately half of the \textit{A. baumannii} strains possessed 16:1 $\omega$9c fatty acids in small amount, but was absent in all of the \textit{A. calcoaceticus} strains. Although there were several fatty acids that show some distinctions, the whole fatty acid profiles of \textit{A. baumannii} and \textit{A. calcoaceticus} are too similar to be discriminated by some specific CFA compositions\textsuperscript{[27]}

\textit{A. baumannii} Subgrouping by Fatty Acid Markers

Two clusters were clearly observed by plotting the ratios Sum In Feature 3/16:0 versus 17:0 and Sum In Feature 3/2-OH-12:0 versus 17:0 fatty acids, indicating that these ratios could serve as chemical markers to differentiate the two \textit{Acinetobacter} strains. Although the characteristics and significance of each cluster are not understood, the distinct classification in \textit{A. baumannii} strains could be further investigated. This phenomenon is presumed to be related to some phenotype, such as drug resistance. However, the characteristics of the samples used in the present research are not complete. Thus, no conclusion can be drawn at present.

Chemical Markers for Differentiating \textit{A. baumannii} and \textit{A. calcoaceticus}

A previous report has revealed that \textit{Yersinia
pestis and two other human-pathogenic Yersinia (Y. pseudotuberculosis and Y. enterocolitica) could be separated by plotting the ratios of the 12:0/16:0 and 14:0/16:0 fatty acids[29]. There are only a few previous studies on the CFAs of Acinetobacter strains. In the present study, the Sherlock MIS was used to analyze the fatty acid compositions of the different strains of A. baumannii and A. calcoaceticus to determine stable chemical markers to differentiate them. Results show that A. baumannii could be easily differentiated from A. calcoaceticus by plotting the ratios of the Sum In Feature 3/18:1 ω9c versus 16:0/18:1 ω9c and unknown 12.484/18:1 ω9c versus Sum In Feature 3/18:1 ω9c fatty acids. Two clusters were clearly observed for each of the two species, indicating that these CFA ratios could serve as a potential method to differentiate A. baumannii from A. calcoaceticus.

In conclusion, there is no significant difference in the CFA profiles between A. baumannii and A. calcoaceticus strains isolated from different locations in China. A. baumannii can be easily classified into two subgroups by plotting some ratios of CFAs. Although the CFA contents are similar in A. baumannii and A. calcoaceticus, two chemical markers were obtained to discriminate these species.

REFERENCES