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Differentiation of *Bordetella avium* and Related Species by Cellular Fatty Acid Analysis

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The fatty acids of 18 strains of *Bordetella avium*, 3 strains of *Alcaligenes faecalis*, 5 strains of *Bordetella bronchiseptica*, and 12 strains of a *B. avium*-like organism were examined by gas chromatography-mass spectrometry. The presence of a significant amount of the acid 2-OH $C_{14:0}$ characterized *B. avium* and the *B. avium*-like organism. *B. avium* and the *B. avium*-like organism differed in their relative concentrations of $C_{16:1}$ and 3-OH $C_{14:0}$ acids. *B. bronchiseptica* and *A. faecalis* were distinguishable by comparison of the relative concentrations of $C_{18:0}$ and $C_{18:1}$ acids.

In the late 1970s a gram-negative, motile, asaccharolytic bacterium was associated with an acute respiratory disease of turkey poults (5). The disease has since been reported by other workers, although different terms were used, e.g., rhinotracheitis (14) and bordetellosis (6).

Considerable confusion and disagreement have occurred concerning the classification of the disease agent. It has been termed *Bordetella*-like (6), *Alcaligenes faecalis* (15), and *Bordetella meleagridis* or *Alcaligenes meleagridis* (13). Kersters et al. (11) demonstrated that the agent is a member of the genus *Bordetella* and proposed the new species *Bordetella avium*. In addition, recent studies have indicated that an organism very similar to *B. avium* also occurs in chickens and turkeys (1, 7, 13; P. J. Blackall and C. M. Doheny, Aust. Vet. J., in press). We have adopted the suggestion of Jackwood et al. (8) and use the term *B. avium*-like for this organism.

A major reason for the confusion over the identification and classification of *B. avium* is that this organism is relatively inert and hence the conventional tests yield mainly negative results. These negative results make separation of *B. avium* from other similar inert organisms such as *A. faecalis* or *Bordetella bronchiseptica* difficult.

In recent years, gas chromatographic profiling of the cellular fatty acid composition of bacterial cells has been successfully used to classify and identify many bacteria (9). In particular, the technique has been used to identify members of the genera *Alcaligenes* (4) and *Bordetella* (3, 10). Recently, Jackwood et al. (8) described, for the first time, the fatty acid profiles of *B. avium* and the *B. avium*-like organism.

This report presents the results of our gas chromatography-mass spectrometry studies of the cellular fatty acid composition of *B. avium*, the *B. avium*-like organism, *A. faecalis*, and *B. bronchiseptica*. They provide some criteria for the separation of these four organisms based on their fatty acid profiles. These criteria are particularly useful for laboratories with access to high-resolution (capillary) gas chromatographs but lacking the software necessary for rapid probability-based identification. In addition, we clarify some ambiguities in the literature concerning the fatty acid profiles of members of the genus *Bordetella*.

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MATERIALS AND METHODS

Cultures. The field isolates of *B. avium* (12 strains) and the *B. avium*-like organism (10 strains) have been described elsewhere (2; Blackall and Doheny, in press). The reference strains used are given in Table 1.

Cell preparation and derivative formation. Bacteria were grown on brain heart infusion agar (GIBCO Laboratories, Brisbane, Australia) with 5% sheep blood for 16 h at 37°C in an atmosphere of 5% CO_2. The confluent growth from three plates was gently harvested in sterile distilled water and washed once, and the cells were resuspended in 3 ml of sterile distilled water divided into 1-ml aliquots, and stored at −20°C. The fatty acid methyl esters were then prepared (12).

Gas chromatography-mass spectrometry. Gas chromatographic-mass spectrometric analysis was performed on a Finnigan 1020B spectrometer equipped with a data system and a National Bureau of Standards library. Chromatography was performed on a DB-5 fused silica column (30 m by 0.25 mm) (J & W Scientific Inc., Rancho Cordova, Calif.) interfaced directly to the mass spectrometer ion source. Injector and interface ovens were maintained at 250°C. Injection was splitless (30 s), with an oven temperature of 120°C. After 2 min, the oven temperature was raised by a linear gradient of 7°C/min to 260°C, where it was maintained for 8 min. The total analysis time was 30 min. The carrier gas was helium, with a linear flow rate of 30 cm/s at 120°C. The mass spectrometer was operated in the electron impact mode and scanned from 35 to 350 atomic mass units in 1 s. Identification of peaks was performed by using the library search facility, by comparison with published spectra, or by analysis of authentic material. Quantification was performed by the software by using a retention time window of 800 s, sufficient to include all fatty acid methyl esters from $C_{10:0}$ to $C_{19:0}$. This resulted in over 50 components commonly being included in the quantification with the major components (Table 2), constituting ca. 85% of the total integrated area.

RESULTS

The range of relative concentrations of cellular fatty acids detected in the *B. avium*, *B. avium*-like organism, *A. faecalis* and *B. bronchiseptica* strains are presented in Table 2. We found little difference for the isolates within each of the four groups. The chromatograms of the type strains of the three

TABLE 1. Identification, origins, and sources of reference strains

Organism	Origin	Source[a]
B. avium		
270-80	Goose, Federal Republic of Germany	Hinz
383-78	Turkey, Federal Republic of Germany	Hinz
591-77	Turkey, Federal Republic of Germany	Hinz
P-8	Turkey, United States	Simmons
002	Turkey, United States	Jackwood
197	Turkey, United States	Jackwood
B. avium-like		
128	Turkey, United States	Jackwood
154	Turkey, United States	Jackwood
B. bronchiseptica		
186	Turkey, United States	Jackwood
188	Turkey, United States	Jackwood
ATCC 19395	Dog	ATCC
ATCC 4617	NK[b]	ATCC
NCTC 8344	NK	NCTC
A. faecalis		
ATCC 8750	NK	ATCC
NCIB 9650	NK	UQM
ATCC 19018	NK	Glenfield

[a] Hinz, K.-H. Hinz, Klinik fur Gleflugel der Tierarztlichen, Hannover, Federal Republic of Germany; Simmons, D. G. Simmons, School of Veterinary Medicine, North Carolina State University, Raleigh, N.C.; Jackwood, M. W. Jackwood, Ohio Agricultural Research and Development Center, Wooster, Ohio; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom; UQM, University of Queensland Culture Collection, Brisbane, Australia; Glenfield, Central Veterinary Laboratory, Glenfield, Australia.

[b] NK, Not known.

recognized species are shown in Fig. 1. The peak marked X is an artifact most probably derived from the C_{17} cyclopropane (cyc) acid during esterification.

The cellular fatty acids of the three species and the *B. avium*-like organisms were qualitatively similar. The major fatty acids present were $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, C_{17} cyc, $C_{18:0}$, $C_{18:1}$, and the hydroxy acids 2-OH $C_{12:0}$ and 3-OH $C_{14:0}$. The acids $C_{16:0}$ and C_{17} cyc constituted about 60% of the total cellular fatty acids for all of the isolates examined.

Examination of the cellular fatty acid profiles allowed the four groups of organisms to be clearly spearated. All the *B. avium* and *B. avium*-like isolates had a 2-OH $C_{14:0}$ content which, although small, was much larger than that of any *A. faecalis* or *B. bronchiseptica* isolate. The *B. avium*-like isolates could be separated from *B. avium* by a comparison of the ratios of $C_{16:1}$ and 3-OH $C_{14:0}$ acids. All *B. avium* isolates had a $C_{16:1}$ content lower than that of the closely adjacent 3-OH $C_{14:0}$, whereas all the *B. avium*-like isolates had a $C_{16:1}$ content higher than the 3-OH $C_{14:0}$ content.

B. bronchiseptica and *A. faecalis* were most easily separated by comparison of the ratios of $C_{18:0}$ and $C_{18:1}$ acids. The three *A. faecalis* isolates showed a $C_{18:1}$ content greater than the $C_{18:0}$ content. For the five *B. bronchoseptica* isolates, the reverse was true, i.e., the $C_{18:0}$ content was higher than the $C_{18:1}$ content. All the isolates of *B. avium* and the *B. avium*-like organisms also had a $C_{18:0}$ content greater than the $C_{18:1}$ content.

The means of the concentrations of the fatty acids useful in separating the four taxa (3-OH $C_{14:0}$, $C_{16:1}$, $C_{18:1}$, and $C_{18:0}$) are presented in Table 3.

DISCUSSION

The cellular fatty acid contents, as determined by gas chromatography-mass spectrometry, allowed the ready recognition of the four taxa examined in this study, *B. avium*, the *B. avium*-like organism, *A. faecalis*, and *B. bronchiseptica*. Although the four taxa all had $C_{16:0}$ and C_{17} cyc as the major fatty acids, the ratios of the minor components clearly separated the organisms (Table 2 and Fig. 1).

Our findings are, in general, in agreement with those of a similar study performed by Jackwood et al. (8). In both studies, *B. avium* and the *B. avium*-like organism were readily distinguished from *A. faecalis* and *B. bronchiseptica* by the presence of significant amounts of 2-OH $C_{14:0}$. However, Jackwood et al. (8) based their differentiation of *B. avium* and the *B. avium*-like organism on statistically significant differences between their $C_{16:0}/C_{14:0}$, $C_{16:0}/C_{18:0}$, and 2-OH $C_{14:0}$/2-OH $C_{12:0}$ ratios and by use of the Hewlett-Packard 5898A microbial identification system. In contrast, our results suggest a much more convenient distinguishing feature: *B. avium* isolates had a $C_{16:1}$ content lower than that of 3-OH $C_{14:0}$, whereas *B. avium*-like isolates had a $C_{16:1}$ content higher than that of 3-OH $C_{14:0}$.

Our examination of 18 *B. avium* isolates and 12 *B. avium*-like isolates from three continents showed the $C_{16:1}$/3-OH $C_{14:0}$ ratio to be a simple and completely reliable method of separating *B. avium* and the *B. avium*-like organism. We emphasize that this subtle but significant difference between *B. avium* and the *B. avium*-like organism necessitates strict adherence to our conditions of growth and chemical workup. Further work on the effects of various incubation conditions and different media is planned.

In agreement with Jackwood et al. (8), we found that the avian isolates of *B. bronchiseptica* possessed fatty acid profiles very similar to those of the reference strains obtained from other animals.

There has been some disagreement concerning the $C_{18:0}$ and $C_{18:1}$ content of members of the genus *Bordetella*. In the study by Jantzen et al. (10) of 13 *Bordetella pertussis* isolates, 3 *Bordetella parapertussis* isolates, and 7 *B. bronchiseptica* isolates, the chromatograms illustrated show a $C_{18:0}$ content greater than the $C_{18:1}$ content. However, in

TABLE 2. Cellular fatty acid composition of strains of *B. avium*, the *B. avium*-like organism, *B. bronchiseptica*, and *A. faecalis*

Organism (no. of strains)	% of total fatty acids											
	$C_{12:0}$	2-OH $C_{12:0}$	$C_{14:0}$	2-OH $C_{14:0}$	3-OH $C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	C_{17} cyc	$C_{18:0}$	$C_{18:1}$
B. avium (18)	0.4–1.0	1.4–2.1	0.6–0.9	1.5–2.6	3.0–4.6	0.1–0.4	28.0–36.0	0.8–2.8	0.8–2.1	19.0–30.0	7.0–13.0	0.2–0.7
B. bronchiseptica (5)	0.5–1.0	1.5–3.7	4.3–7.2	<0.2	3.5–5.3	0.4–1.4	35.0–42.0	6.0–17.0	0.9–2.0	14.0–22.0	3.0–7.0	1.2–2.3
A. faecalis (3)	1.0–2.0	1.0–2.5	0.5–1.5	<0.2	4.3–8.2	0.2–1.2	34.0–40.0	6.5–14.0	0.8–1.0	15.0–23.0	1.5–2.7	5.0–9.0
B. avium-like (12)	0.4–1.1	1.0–3.3	0.6–1.3	1.5–4.0	3.4–5.1	0.2–0.4	31.0–38.0	5.5–12.0	0.9–2.4	11.0–23.0	5.0–9.0	1.0–2.7

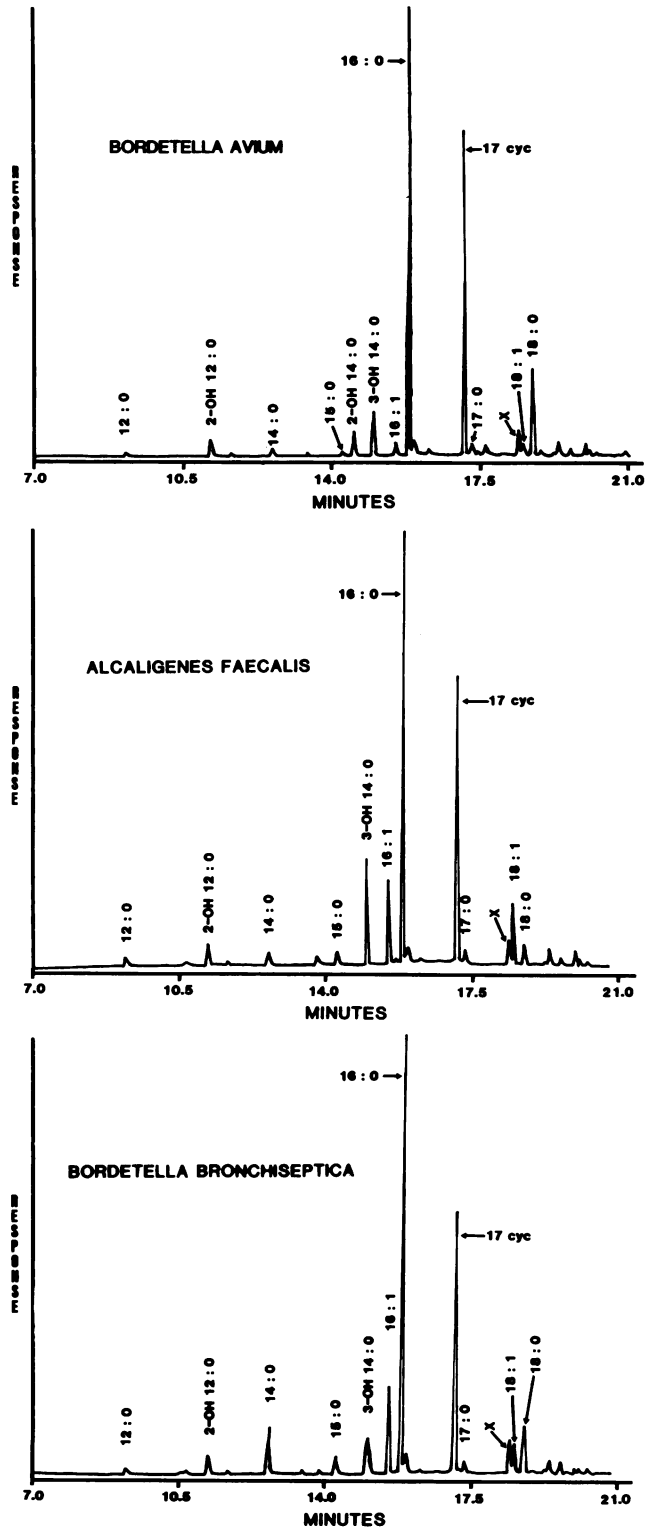

FIG. 1. Chromatograms of *B. avium* 591-77, *A. faecalis* ATCC 8750, and *B. bronchiseptica* ATCC 19395. For conditions, see the text. The instrument software normalized the chromatogram automatically to the major component, $C_{16:0}$.

TABLE 3. Concentrations of fatty acids used in ratio comparisons

Organism (no. of isolates)	Mean concn (%) of:			
	3-OH $C_{14:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$
B. avium (18)	3.7	1.6	10.7	0.4
B. bronchiseptica (5)	4.1	10.7	4.4	1.6
A. faecalis (3)	6.2	9.0	2.0	6.5
B. avium-like (12)	4.2	9.1	8.0	2.0

the table accompanying the chromatograms, the values for *B. bronchiseptica* and *B. parapertussis* indicate a reverse ratio, i.e., $C_{18:1}$ content greater than $C_{18:0}$ content. Dees et al. (3) reported similar levels of $C_{18:0}$ and $C_{18:1}$ in five isolates of *B. bronchiseptica*. Jackwood et al. (8) reported that 13 *B. bronchiseptica* isolates and 5 *B. parapertussis* isolates were all characterized by a $C_{18:1}$ content greater than the $C_{18:0}$ content. However, for 59 *B. avium* isolates and 46 *B. avium*-like isolates, Jackwood et al. (8) reported the opposite ratio, i.e., $C_{18:0}$ content greater than $C_{18:1}$ content. In contrast, we found that all *Bordetella* isolates we examined (18 *B. avium* isolates, 5 *B. bronchiseptica* isolates, and 12 *B. avium*-like isolates) had a $C_{18:0}$ content greater than the $C_{18:1}$ content.

It is possible that peak X in Fig. 1, clearly identifiable as an artifact by mass spectrometry and most probably derived from C_{17} cyc during esterification, may have contributed to this confusion concerning the $C_{18:0}/C_{18:1}$ ratio. The identities and origins of this and similar artifacts have been described by Vulliet et al. (16). The major fragments in its mass spectrum are at *m/e* 129 and *m/e* 201. Laboratories basing fatty acid identification on retention time data could misidentify this artifact as a $C_{18:1}$ isomer. Our results show that a $C_{18:1}$ isomer was occasionally detectable superimposed on the artifact. In such instances, it was present only in trace quantities (<0.2%).

It is likely that for bacterial species in which the C_{17} cyc component is at a low level or absent, no ambiguity due to the artifact would arise. This may explain why Jantzen et al. (10) reported that the $C_{18:0}$ content of *B. pertussis*, an organism containing only trace levels of C_{17} cyc, was greater than the $C_{18:1}$ content.

In species in which the $C_{18:0}$ component is sufficiently large, the mistaken inclusion of the artifact as a $C_{18:1}$ isomer would not result in a $C_{18:1}$ content greater than the $C_{18:0}$ content. This may explain why Jackwood et al. (8) reported that all their *B. avium* and *B. avium*-like isolates had a $C_{18:0}$ content greater than the $C_{18:1}$ content yet reported the opposite for their *B. bronchiseptica* and *B. parapertussis* isolates.

In contrast to our results for the three *Bordetella* taxa, we found that the three *A. faecalis* isolates had the reverse ratio of $C_{18:0}$ and $C_{18:1}$ acids, i.e., a $C_{18:1}$ content greater than the $C_{18:0}$ content. Previous studies of 29 *A. faecalis* isolates, 4 *Alcaligenes denitrificans* isolates, and 11 "*Alcaligenes odorans*" isolates have all reported a similar finding (3, 4, 8).

Our results suggest that when isolates of *A. faecalis*, *B. bronchiseptica*, *B. avium*, and the *B. avium*-like organism are examined for cellular fatty acids by using the growth medium and conditions we describe, the ratios of $C_{18:0}$ and $C_{18:1}$ fatty acid contents provide a clear distinction between the two genera.

As also reported by Jackwood et al. (8), we found that all of our isolates of *B. avium* and the *B. avium*-like organism possessed significant amounts of 2-OH $C_{14:0}$. In contrast, our

A. faecalis and *B. bronchiseptica* isolates possessed only trace amounts of this acid. Other studies have confirmed the absence of this acid in other members of the genus *Bordetella* (3, 8, 10).

Small amounts of 2-OH $C_{14:0}$ have been found in some isolates of *A. faecalis* (4, 8). This finding would still not confuse the identification of *B. avium* or the *B. avium*-like organism. The ratio of the $C_{18:0}$ and $C_{18:1}$ content would still clearly separate any 2-OH $C_{14:0}$-containing *A. faecalis* isolates from *B. avium* or the *B. avium*-like organism.

In summary, we confirmed that the four taxa, *B. avium*, the *B. avium*-like organism, *B. bronchiseptica*, and *A. faecalis* possess distinct cellular fatty acid profiles. The three members of the genus *Bordetella* could be distinguished from *A. faecalis* by comparison of the relative amounts of $C_{18:0}$ and $C_{18:1}$ acids present. *B. avium* and the *B. avium*-like organism contained the acid 2-OH $C_{14:0}$ at much higher levels than did *B. bronchiseptica* or *A. faecalis*. *B. avium* and the *B. avium*-like organism could be separated by a comparison of the relative amounts of $C_{16:1}$ and 3-OH $C_{14:0}$ acids. For diagnostic veterinary laboratories with access to a high-resolution gas chromatograph, the detection of cellular fatty acid profiles offers a rapid and reliable method for the identification of *B. avium* and the *B. avium*-like organism.

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