Cellular Fatty Acid Composition Comparisons of *Haemophilus equigenitalis* and *Moraxella* Species

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The cellular fatty acid compositions of *Haemophilus equigenitalis* and some other species were compared. The cellular fatty acid composition of *H. equigenitalis* was very similar to the cellular fatty acid compositions of *Moraxella* species. When the double bond positions in monounsaturated fatty acids were determined by a gas chromatography-mass spectrometry analysis of ditrimethylsilyloxy derivatives, *Moraxella* species were divided into two groups, the oleic acid group and the *cis*-vaccenic acid group. The former included eight species (*Moraxella atlantae, Moraxella bovis, Moraxella caviae, Moraxella equi, Moraxella lacunata, Moraxella nonliquefaciens, Moraxella osloensis, and Moraxella phenylpyruvica*), and the latter included *Moraxella catarrhalis, Moraxella ovis,* and *Moraxella urethralis.* *H. equigenitalis* was closely related to the *cis*-vaccenic acid group.

The taxonomic position of *Haemophilus equigenitalis*, the causative agent of contagious equine metritis, is still unclear because this organism does not require X- or V-factors (7, 29, 31). Recently, we studied the cellular fatty acid composition of *H. equigenitalis* and found that it is characterized by large amounts of 18:1 and 16:0 acids (30). The cellular fatty acid composition of this species is grossly similar to the cellular fatty acid compositions of *Moraxella* species (5, 9, 14, 15, 18, 27). In addition, *H. equigenitalis* is catalase and oxidase positive, asaccharolytic, and very unreactive in the conventional biochemical tests (31) and thus is phenotypically similar to species of the genus *Moraxella* (12). However, guanine-plus-cytosine content of deoxyribonucleic acid (DNA) from this organism (36.1 mol% [31]) is distinctly lower than the range of values found in the genus *Moraxella* (4). In this study, the cellular fatty acid composition of *H. equigenitalis* was compared with the cellular fatty acid compositions of *Moraxella* species and species of other related genera to clarify possible relationships among these species.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The strains used in this study are listed in Table 1. The bacterial cells were cultivated on Eugon agar supplemented with 5% Fildeps pepsin-digested sheep blood for 72 h under microaerophilic conditions. The cells were freeze-dried after they were washed three times with saline.

**Extraction and methylation of fatty acids.** The dried cells were treated with 5% HCl in methanol at 105°C for 3 h, and fatty acid methyl esters were extracted with hexane as previously described (21).

**Determination of double bond positions in monounsaturated fatty acids.** The monounsaturated fatty acid methyl esters in each mixture were converted to the corresponding dihydroxy fatty acids by reaction with hexamethyldisilazane and trimethylchlorosilane in pyridine (TMS-HT kit; Tokyo Chemical Industry Co. Ltd., Tokyo, Japan). The dihydroxy fatty acids were subsequently converted to the corresponding ditrimethylsilyloxy ethers by reaction with a gas chromatograph-mass spectrometer-computer system (model 5992B; Hewlett-Packard) equipped with a glass capillary column (0.28 mm by 30 m) coated with OV-101, GC-MS was performed by using a gas chromatograph-mass spectrometer-computer system (model 5992B; Hewlett-Packard) equipped with a glass capillary column (0.28 mm by 30 m) coated with OV-101. Fatty acid methyl esters were analyzed at an oven temperature of 180°C, and the dihydroxy fatty acids were analyzed at 200°C.

**Identification of fatty acids.** Fatty acids were identified primarily by equivalent chain length (ECL) calculations, using a standard mixture of fatty acid methyl esters, as previously described (22). The methyl esters of oleic acid (Δ9 18:1 acid) and *cis*-vaccenic acid (Δ11 18:1 acid) were also used as monounsaturated fatty acid standards. All structural determinations of fatty acids were verified by GC-MS.

**Numerical analysis.** The similarities among the strains tested based on cellular fatty acid composition
TABLE 1. Strains used in this study

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* Sources: 1, American Type Culture Collection, Rockville, Md.; 2, R. Sakazaki, National Institute of Health, Tokyo, Japan; 3, National Collection of Type Cultures, London, England; 4, Y. Akiyama, Tochigi Branch Laboratory, Equine Health Laboratory, Tochigi, Japan (originally supplied by J. T. Bryans, College of Agriculture, University of Kentucky, Lexington); 5, our collection.

b Type strain.

were calculated by using the formula of Bergan (2), as previously described (21).

RESULTS

The cellular fatty acid compositions of Moraxella, Kingella, and Legionella species and H. equigenitalis NCTC 11184 are shown in Table 2. Species belonging to the genus Moraxella generally contained large amounts of 16:0 and 18:1 acids, and the fatty acid compositions of these species were grossly similar to the fatty acid composition of H. equigenitalis (30). Kingella kingae contained 14:0, 16:1, and 16:0 acids as the major fatty acids and produced a fatty acid pattern that was different from the patterns of Moraxella species and H. equigenitalis. Moraxella anatipestifer and Legionella pneumophila contained large amounts of branched-chain fatty acids, especially iso-15:0 acid, and produced fatty acid patterns that were distinctly different from those of the other species tested.

The ECL values of the 18:1 acid indicated that positional isomers might exist. Because the methyl esters of positional isomers produce very similar mass spectra, the positions of double bonds could not be determined directly from the spectra. To determine the double bond positions, monounsaturated fatty acid methyl esters were converted to the corresponding trimethylsilyl oxide derivatives and then analyzed by GC-MS. GLC patterns of fatty acid methyl esters of H. equigenitalis before and after derivation are shown in Fig. 1 as examples. After derivation, the peak of the 16:1 acid completely disappeared, whereas a reduced amount of the 18:1 acid was still detected. Two new peaks, which appeared at retention times of about 17.5 and 33 min, were thought to correspond to the trimethylsilyl oxide derivatives of 16:1 and 18:1 acids, respectively. The mass spectra of the trimethylsilyl oxide derivatives of monounsaturated fatty acid methyl esters detected in H. equigenitalis and M. bovis, are shown in Fig. 2. These spectra show a characteristic cleavage between two trimethylsilyloxy groups, resulting in very simple mass spectra that are dominated by the two cleavage fragments, as shown in Fig. 3 (6). The peak at m/e 73 was due to the trimethylsilyl ion. H. equigenitalis and M. urethralis contained Δ11 18:1 acid and palmitoleic acid (Δ9 16:1 acid), and H. equigenitalis also contained a trace of Δ9 18:1 acid. M. bovis and M. osloensis contained Δ9 18:1 and Δ9 16:1 acids, and Δ9 17:1 acid was detected in M. bovis. The double bond positions of 18:1 acids detected in the other species, which were not included in our determination of double bond positions by trimethylsilyloxy derivatization, were easily deduced from their ECL values. The ECL value of Δ11 18:1 acid was 17.77 ± 0.01, and that of Δ9 18:1 acid was 17.71 ± 0.01.

After we calculated the correlation coefficient for all of the strains, we constructed a dendrogram based on cellular fatty acid composition (Fig. 4). Moraxella species formed two clusters. The first cluster included Moraxella catarrhalis, Moraxella ovis, and M. urethralis, which contained cis-vaccenic acid as a major fatty acid. The strains of H. equigenitalis were very similar to each other and were closely related to the cis-vaccenic acid group of Moraxella species. The other Moraxella species (Moraxella atlantae, M. bovis, Moraxella caviae, Moraxella equi, Moraxella lacunata, Moraxella nonliquefaciens, M. osloensis, and Moraxella phenylpyruvica) formed the oleic acid group. M. anatipestifer and L. pneumophila were separated from the two clusters of Moraxella species. K. kingae could be distinguished from the Moraxella species by cellular fatty acid composition.

DISCUSSION

We previously studied the cellular fatty acid composition of H. equigenitalis (30) and found...
TABLE 2. Cellular fatty acid compositions of the strains tested.

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<th>Serial number</th>
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The number to the left of the colon indicates the number of carbon atoms; the number to the right of the colon indicates the number of double bonds. Fatty acids that accounted for less than 0.5% of the total are not shown.

- \( a \) indicates a methyl branch at the anteiso and iso carbon atoms, respectively.
- \( 30H \) indicates the position of the hydroxy group.
- A indicates the position of a double bond.
that it is clearly distinct from the cellular fatty acid compositions of *Haemophilus* species (13) and grossly similar to the cellular fatty acid compositions of *Moraxella* species (5, 9, 14, 15, 27). *Moraxella* species have been reported to contain large amounts of 16:0 and 18:1 acids, which we confirmed in this study. The cellular fatty acid composition of *M. anatipestifer*, whose biochemical characteristics are markedly different from those of other *Moraxella* species (1), was peculiar in its abundance of branched-chain fatty acids and was similar to the cellular fatty acid compositions of *Legionella* species (23, 24, 26).

Bøvre and colleagues studied the cellular fatty acid compositions of *Moraxella* and *Neisseria* species (5, 9, 14, 15) and found that false *Neisseria* or *Branhamella* (i.e., *M. catarrhalis, M. caviae*, and *M. ovis*) strains have fatty acid patterns grossly similar to those of *Moraxella* species. This observation is one of the reasons that the false *Neisseria* species were reclassified in the genus *Moraxella*. Bøvre described the taxonomic problems encountered with the false *Neisseria* species and proposed to divide the genus *Moraxella* into the following two subgenera, based mainly on cell shape: subgenus *Moraxella*, containing rod-shaped organisms, and subgenus *Branhamella*, containing cocci (3).

However, Bøvre et al. did not distinguish positional isomers of 18:1 acid, which is a major fatty acid in *Moraxella* species. Two species of subgenus *Branhamella*, *M. catarrhalis* and *M. ovis*, were clearly separated from species belonging to subgenus *Moraxella* and *M. caviae* by the positional isomer compositions of 18:1 acids in the present study. The positional isomers of 18:1 acid have taxonomic significance in the genus *Moraxella* since this genus has been divided into the oleic acid group and the *cis*-vaccenic acid group. The double bond positions in 18:1 acids were easily deduced from ECL values when a GLC analysis was performed by using a flexible fused capillary column because of its...
FIG. 2. Mass spectra of ditrimethylsilyloxy derivatives of monounsaturated fatty acid methyl esters. Δ9 16:1 acid (A) and Δ11 18:1 acid (B) were detected in *H. equigenitalis*, and Δ9 18:1 acid (C) was detected in *M. bovis*.

high resolution and separation capacity, as reported by Moss et al. (25).

In contrast to the previous finding that *M. urethralis* is separated from other *Moraxella* species by cellular fatty acid composition (14, 15), *M. urethralis*, *M. catarrhalis*, and *M. ovis* clustered together in our study. This apparently resulted from differences in the methods used to calculate similarity. The primary logarithmic transformation of the data for each fatty acid used in the previous studies may have given relatively more weight to fatty acids that occur in small quantities. For example, *M. urethralis* contained relatively small amounts of 14:0 and 3OH 14:0 acids but lacked 10:0, 12:0, and 3OH 16:0 acids, in contrast to *M. catarrhalis* and *M. ovis*. Furthermore, the amount of 16:1 acid was relatively smaller than the amount of 16:0 acid in *M. urethralis* compared with the other two species.

It is known that unsaturated fatty acids are synthesized from saturated fatty acids by two pathways (aerobic and anaerobic) (8, 10, 11). The anaerobic pathway of desaturation generally leads to palmitoleic and cis-vaccenic acids as the major products, whereas the aerobic pathway yields primarily palmitoleic and oleic acids (10). The detection of Δ9 monounsaturated fatty acids in *M. bovis* and *M. osloensis* strongly suggests that these organisms have the aerobic pathway of desaturation at the Δ9 position. The existence of this pathway in gram-negative bacteria is known only in *Alcaligenes faecalis*, although it is common in gram-positive bacteria (17). On the other hand, the detection of ω7 monounsaturated fatty acids in *H. equigenitalis* and *M. urethralis* indicates the existence of the anaerobic pathway of desaturation. *H. equigenitalis* synthesizes a trace amount of oleic acid, probably by the anaerobic pathway since the two pathways are mutually exclusive in the sense that no organism which possesses both

\[ m/e = 14\omega + 89 \]

\[ CH_2(CH_2)_\omega-\omega COOCH_3 \]

\[ CH(CH_2)_\omega-\omega COOCH_3 \]

\[ m/e = 14\omega + 133 \]

FIG. 3. Typical cleavage of ditrimethylsilyloxy derivatives (OTMS) of a monounsaturated fatty acid methyl ester. Δ and ω indicate the numbers of carbon atoms measured from the terminal methyl base and carboxyl base, respectively.
pathways has been found (8, 11).

In our previous study of H. equigenitalis (30), we found no significant difference in cellular fatty acid composition among cells cultivated on different media even if we substituted a chemically defined growth supplement, such as IsoVitalex (BBL Microbiology Systems, Cockeysville, Md.), for blood components. However, we tested in this study incorporate fatty acids from the medium, as indicated by Letts et al. (20). If this is the case, the differences in cellular fatty acid composition result from differences in the ability to incorporate lipid constituents from the medium rather than differences in pathways of desaturation. Because only a few species contain both of oleic and cis-vaccenic acids and either of these acids is dominant in amount, the possibility that the dominant positional isomer was incorporated from the medium is unlikely. However, the pathway by which organisms synthesize monounsaturated fatty acids must be proved directly by using labeled precursors.

Taylor et al. proposed that the causative organism of contagious equine metritis should be classified as a member of the genus Haemophilus based on the guanine-plus-cytosine content of its DNA and its X-factor requirement, and these authors designated this organism H. equigenitalis (31). The X-factor requirement of this organism was reported by Shreeve (28), but Taylor et al. (31), Dabernat et al. (7), and we could not confirm this result. Because the porphyrin test for this organism is positive, like other X-factor-independent Haemophilus species (16), this organism does not require X-factor. H. equigenitalis is a catalase- and oxidase-positive ascaccharolytic coccobicillus, is very unreactive in conventional biochemical tests, and shows phenotypic similarity to Moraxella and Legionella species. In cellular fatty acid composition, H. equigenitalis is very similar to the cis-vaccenic acid group of Moraxella species but distinct from the oleic acid group of Moraxella species and Legionella species.

When other taxonomic criteria are taken in consideration, H. equigenitalis differs from the Moraxella species described above in some characteristics. The guanine-plus-cytosine contents of M. urethralis, M. catarralalis, M. ovis, and H. equigenitalis DNAs are 46 to 47, 46.5 to 47.5, 44.5 to 46.5, and 36.1 mol%, respectively (4, 31). Furthermore, M. urethralis, which has been tentatively placed in the genus Moraxella despite genetic incompatibility with this genus, grows slowly in a simple minimal medium supplemented with ammonium ions as the source of nitrogen and acetate or hydroxybutylate as the carbon source (19). The nutritional properties of H. equigenitalis, which have not been reported, seem rather complex.

Further taxonomic studies based on DNA-DNA hybridizations and cellular enzyme profiles are now in progress to clarify the possible relationship of H. equigenitalis to the family Neisseriaceae.

ACKNOWLEDGMENT

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LITERATURE CITED


