Cellular Fatty Acid and Soluble Protein Composition of *Actinobacillus actinomycetemcomitans* and Related Organisms

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The cellular fatty acid and protein content of twenty-five representative strains of *Actinobacillus actinomycetemcomitans* isolated from juvenile and adult periodontitis patients was compared to that of 15 reference strains of oral and nonoral *Actinobacillus* species and *Haemophilus aphrophilus*. Trimethylsilyl derivatives of the fatty acid methyl esters were analyzed by gas-liquid chromatography. The predominant fatty acids of all 40 strains examined were 14:0, 3-OH 14:0, 16 Δ , and 16:0. *Actinobacillus seminis* (ATCC 15768) was unlike the other strains examined because of a greater amount of 14:0 detected. The soluble protein analysis using polyacrylamide gel electrophoresis revealed that *A. actinomycetemcomitans*, *H. aphrophilus*, and nonoral *Actinobacillus* species possessed distinct protein profiles attesting to the validity of separating these organisms into different species. Established biotypes of *A. actinomycetemcomitans* could not be differentiated on the basis of fatty acid or protein profiles.

Identification and characterization of microorganisms has historically been based on their morphological, biochemical, serological, toxigenic, and genetic characteristics. More recently, gas chromatographic analysis of cellular fatty acids and examination of cytoplasmic protein patterns using polyacrylamide gel electrophoresis have been used taxonomically to distinguish among strains within a species (5) and among organisms within a genus or family (1, 4, 16). Grouping microorganisms on the basis of cellular fatty acid and protein content has generally correlated well with results from genetic and numerical taxonomic studies.

Actinobacillus actinomycetemcomitans (*Bacterium actinomycetem comitans*) was first described by Klinger in 1912 (11). The initial description of this gram-negative facultative organism isolated from actinomycotic lesions was meager, listing only morphological and cultural characteristics. In 1929, Topley and Wilson transferred the organism to the genus *Actinobacillus* with little amplification of the description (19). King and Tatum in 1962 (10) presented the first detailed biochemical and serological description of the species, attempting to clarify the scant and confusing reports in the literature and to distinguish the actinobacillus from the morphologically similar *Haemophilus aphro-*

philus. The 33 strains studied were isolated from blood, actinomycosis, and respiratory tract, face, neck, and jaw infections. In a study of the fermentative capabilities of 140 strains of *A. actinomycetemcomitans*, Pulverer and Ko (17) identified eight biotypes on the basis of variable fermentation of galactose, mannitol, and xylose. Slots et al. (18) recently associated *A. actinomycetemcomitans* with juvenile periodontitis and established 10 biotypes based on variable fermentation of dextrin, maltose, mannitol, and xylose. In the 8th edition of *Bergey's Manual of Determinative Bacteriology* (15), however, *A. actinomycetemcomitans* is listed as species incertae sedis, indicating that the taxonomic position of this organism is still not adequately defined.

Because of the increasing realization that *A. actinomycetemcomitans* is a potentially pathogenic organism in humans, this study examined the cellular fatty acid and soluble protein content of *A. actinomycetemcomitans*, other *Actinobacillus* species, and *H. aphrophilus* to better delineate the taxonomic relatedness of these organisms.

MATERIALS AND METHODS

Microorganisms. The 15 reference strains and the 25 *A. actinomycetemcomitans* strains representing 10 biotypes, the sites from which they were isolated, and

the sources from which they were obtained are presented in Table 1. Classification of the isolates was carried out using established procedures (3, 6).

All strains were maintained by weekly transfer on tryptic soy agar (Difco Laboratories, Detroit, Mich.) supplemented with 1.0 g of yeast extract per liter and 50 ml of defibrinated sheep blood per liter in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) at 37°C with 85% N₂-10% H₂-5% CO₂. Bacterial cells which were utilized for protein and fatty acid analysis were grown in the anaerobic chamber in 200 ml of brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1.0 g of yeast extract per liter. After 48 h, cells were harvested by centrifugation, washed twice with phos-

phate-buffered saline (pH 7.0), and stored at -20°C until analyzed.

Fatty acid analysis. The method of Mayberry (12) was used to obtain the fatty acid profiles of the strains. The cell pellets were suspended in 2.5 ml of 2 N HCl containing 0.5 µmol of nonadecanoic acid as an internal standard, and either hydrolyzed overnight at 100°C or autoclaved for 1 h at 121°C in Teflon-lined, screw cap tubes. Hydrolysates were extracted once with 5 ml of chloroform, and the aqueous layers were discarded. The organic layers were treated with 2.5 ml of 1 N HCl in methanol for 30 to 60 min at 100°C to methylate the fatty acids. After cooling, the mixtures were partitioned and washed three times with 5-ml portions of distilled water, and the final chloroform

TABLE 1. *List of strains*

Organism	Strain	Source ^a	Site of origin ^b
<i>A. actinomycetemcomitans</i>	29522	ATCC	Mandibular abscess
	29523	ATCC	Blood
	29524	ATCC	Chest aspirate
	9709	NCTC	Lumbar abscess
	9710	NCTC	Lung abscess
	Y4	FDC	JP
	1	Slots	Healthy juvenile-tongue
	14	Slots	JP-NP
	26	Slots	JP-tongue
	28	Slots	Healthy adult-NP
	30	Slots	JP-DP
	34	Slots	Healthy adult-NP
	37	Slots	Healthy adult-NP
	39	Slots	Healthy adult-cheek mucosa
	45	Slots	JP-NP
	55	Slots	JP-cheek mucosa
	56	Slots	JP-DP
	64	Slots	AP-NP
	68	Slots	AP-DP
	73	Slots	AP-NP
	74	Slots	AP-DP
	76	Slots	JP-DP
	78	Slots	JP-cheek mucosa
	79	Slots	Healthy juvenile-NP
	81	Slots	JP-DP
	88	Slots	JP-cheek mucosa
	93	Slots	JP-DP
	97	Slots	AP-DP
	112	Slots	AP-DP
	117	Slots	Healthy adult-NP
	120	Slots	JP-DP
<i>A. seminis</i>	15768	ATCC	Ovine semen
<i>A. suis</i>	15557	ATCC	Blood-adult swine
<i>A. equuli</i>	19392	ATCC	Blood-foal
<i>A. lignieresii</i>	19393	ATCC	Bovine lesion
<i>H. aphrophilus</i>	13252	ATCC	Unknown
	19415	ATCC	Endocarditis
	5906	NCTC	Endocarditis
	5907	NCTC	Endocarditis
	5908	NCTC	Endocarditis

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, England; FDC, Forsyth Dental Clinic, Boston, Mass.; Slots, J. Slots, State University of New York at Buffalo, New York.

^b Abbreviations: JP, juvenile periodontitis; AP, adult periodontitis; DP, deep periodontal pocket; NP, normal periodontal area.

extract was evaporated to dryness under N_2 . The residue was treated with 0.2 ml of a trimethylsilylation mixture consisting of pyridine-hexamethyldisilazane-trimethylchlorosilane-*N,O*-bistrimethylsilyltrifluoroacetamide (2:2:1:1, vol/vol; 13) at room temperature for 15 to 30 min. Immediately before analysis, the reagent was removed under a stream of N_2 , the residue was taken up in 1.0 ml of hexane or dichloromethane, and 0.2 to 0.5 μ l was injected for gas-liquid chromatographic analysis.

Chromatography was carried out with a Hewlett-Packard 5840A gas chromatograph equipped with flame ionization detection and electronic integration. Samples were analyzed on a 10-m capillary column coated with the nonpolar liquid phase SP-2100 (Supelco, Inc., Bellefonte, Pa). Chromatographic conditions were those previously described (12). Peaks were identified by their equivalent chain length values as compared to standards (12). Identification of the hydroxy fatty acids was confirmed by gas-liquid chromatography after conversion of the trimethylsilyl derivatives to trifluoroacetate derivatives by treating the sample with 0.5 ml of trifluoroacetic anhydride-dichloromethane (1:1, vol/vol) at 100°C for 15 min. Identification of the unsaturated fatty acids was confirmed by gas-liquid chromatography after adding a few drops of Br_2 in chloroform to the sample at room temperature and allowing the mixture to sit for 5 min. This treatment resulted in the disappearance of the unsaturated fatty acid peaks from the chromatogram.

Soluble protein analysis. The polyacrylamide slab gel electrophoresis procedure of Moore et al. (14) was used to compare the cellular proteins of the strains. The bacterial cells from the 200-ml broth culture were suspended in a 15-ml centrifuge tube (Nalgene 3117) which contained 0.2 ml of 0.15 M tris(hydroxymethyl)aminomethane-chloride buffer (pH 7.0) and 0.15 g of 100- to 110- μ m-diameter glass beads (B. Braun Melsungen Apparatebau glasperlen 54140, Bronwill Scientific Inc., Rochester, N.Y.). The bacterial suspension was mixed in a Vortex mixer (VRW Scientific, Rochester, N.Y.; speed control setting 7) for two 2-min periods to break the cells. The cellular debris was removed by centrifugation at 8,000 $\times$ g for 10 min, and the supernatant was transferred to a 0.5-ml polypropylene test tube.

Vertical anionic gel electrophoresis in slab gels (14 by 17 cm) using 0.025 M tris(hydroxymethyl)aminomethane-0.2 M glycine electrode buffer (pH 8.5) was carried out in a model 220 electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.) with an 8.5% polyacrylamide resolving gel and a 4.7% polyacrylamide stacking gel. Approximately 50- μ l portions of sample in 30% sucrose were loaded into the wells, and the gels were subjected to 150 V (constant) for 2.5 h at room temperature. Bromophenol blue was used as a tracking dye.

Gels were fixed with 12% trichloroacetic acid for 20 min at room temperature, stained with 0.08% Coomassie blue stain for 10 min, destained with 10% glacial acetic acid in water, and sealed in a plastic Ziploc bag for photographing and storage. The gels were photographed on a glow box using Polaroid 105 film, an orange filter, and a lens setting of 0.5 s at f/11.

RESULTS

Fatty acid composition. Tables 2 and 3 show the percent composition, expressed as moles percent, of the cellular fatty acids of the 40 strains examined. Two or three analyses were routinely made of each strain; the moles percent values varied by no more than 2% of the average value for each peak. Since the repeat analyses were performed 4 to 6 months apart, it does not appear that the use of different lots of media affected the results. Thirty-nine of the organisms, including all test strains of *A. actinomycetemcomitans*, *H. aphrophilus*, *Actinobacillus suis*, *Actinobacillus equuli*, and *Actinobacillus lignieresii*, had the same overall pattern with 14:0 and 3-OH 14:0 (10 to 25 mol%), and 16 Δ and 16:0 (23 to 39 mol%) as the predominant fatty acids. Relatively little but varying amounts of the 18C unsaturated and saturated acids were also detected. Small amounts of 3-OH 10:0, 12:0, 3-OH 12:0, 15:0, 3-OH 15:0, 3-OH 16:0, and five unidentified fatty acids were observed in the majority of these strains. *Actinobacillus seminis* (ATCC 15768) had a qualitative resemblance to the other 39 strains, with 14:0, 3-OH 14:0, 16 Δ , and 16:0 being the predominant fatty acids. A marked quantitative difference was observed, however, as 14:0 represented 45 mol% of the fatty acids detected in *A. seminis* compared with the 10 to 25 mol% 14:0 in the other 39 study strains.

Soluble protein patterns. The electrophoretic fractionations of the cellular proteins are shown in Fig. 1 and 2. Five analyses were made for each strain, and the polypeptide patterns observed were reproducible. Although some minor qualitative or quantitative differences were observed in the bands of strains of the same species, each species gave a distinct, characteristic pattern of negatively charged proteins which was different from those of the other species tested.

DISCUSSION

The taxonomic position and the pathogenic potential of *A. actinomycetemcomitans* are receiving increasing attention because of this organism's possible role in certain types of human periodontal disease (18). This study reports on the value of cellular fatty acid and soluble protein analysis in differentiating between species of actinobacilli and *H. aphrophilus*.

The fatty acid profiles revealed no systematic qualitative or quantitative differences among the representative study strains of the 10 *A. actinomycetemcomitans* biotypes, between *A. actinomycetemcomitans* and *H. aphrophilus*, or among *A. actinomycetemcomitans* and three of

TABLE 2. Cellular fatty acid profiles of *A. actinomycetemcomitans* strains

Strain	Fatty acid composition (mol%)																
	n12:0 ^a (12.0) ^b	h10:0 (12.3)	14br? (13.7)	n14:0 (14.0)	h12:0 (14.2)	? (14.4)	n15:0 (15.0)	? (15.4)	16A (15.8)	n16:0 (16.0)	h14:0 (16.1)	? (17.1)	h15:0 (17.2)	? (17.3)	18A (17.6)	tr ^c	h16:0 (18.2)
ATCC 29522				17.9					33.3	32.3	16.5						
ATCC 29523			0.1	13.6					29.6	29.1	18.5						
ATCC 29524	tr	0.1		14.8	0.2			1.9	26.2	32.2	19.6		0.5		0.4	0.2	0.6
NCTC 9709	tr	0.3		15.0	0.3			1.5	29.4	32.0	16.7		0.3		0.1	0.1	1.2
NCTC 9710	0.3	0.5		19.3	0.3	0.8			25.7	23.2	25.6				1.0	0.1	2.5
FDC Y4	0.1		0.1	12.6	0.8	0.2		0.1	37.2	30.5	13.4				0.1	0.5	1.5
Slots																	
1			0.1	17.0		0.5			29.9	32.2	18.0				0.2	0.9	0.7
14				12.7		0.6			33.2	33.8	14.3				0.2	1.7	2.5
26	0.3	0.1		18.4	0.1		0.1		33.5	29.0	15.2				0.2	0.6	0.5
28				16.2		0.4			31.0	32.4	16.4				0.1	0.8	1.7
30				18.0		0.6			26.0	31.6	22.3				0.7	0.4	0.5
34	0.2	0.2	0.1	20.2	0.1	0.7	0.2	0.1	28.6	29.7	17.7	0.1	0.1	0.1	0.7	0.5	0.6
37				16.8		0.5			26.3	34.4	18.7				0.5	0.3	2.4
39	tr	tr		13.1	tr			1.3	32.7	30.9	20.6		0.2		0.2	0.8	3.0
45				11.9		0.8			34.3	33.4	15.5				1.3		2.4
55				17.0					23.9	33.7	21.8				0.2	0.8	2.4
56	0.1		0.1	15.9		0.7			30.9	32.0	16.8				0.2	0.8	1.8
64	0.3		0.1	19.0		0.6			32.8	29.6	15.9				0.4	0.6	0.6
68				14.4		0.3			35.5	32.0	14.9				0.4	0.6	1.7
73				16.6					32.5	35.7	14.8						0.5
74	0.2	0.2		19.7	0.2			2.0	30.8	30.8	14.8		0.2		0.3	0.2	0.1
76	0.3	0.2	0.1	19.4	0.1	0.8	0.1	0.1	26.9	30.1	18.8	0.1	0.1	0.2	0.2	0.7	1.1
78	tr	tr		18.3	tr			1.1	31.1	32.4	15.9		0.2		0.2	0.3	0.4
79				16.4					33.3	30.2	19.0				0.3	0.5	0.3
81	0.2	0.1		18.9		0.4	0.2	0.1	32.7	28.2	17.9				0.4	0.6	0.4
88	0.4	0.1	0.2	17.6	0.1	0.7	0.1	0.1	28.3	30.6	17.1	0.1	0.1	0.2	0.3	1.1	1.8
93				10.9					28.1	35.5	25.6				tr		tr
97	0.2			16.6		0.5			35.0	30.9	14.6				0.6	0.7	0.9
112				16.9					26.5	33.1	23.6				tr		tr
117	tr	tr		19.1	tr			1.4	29.3	30.0	17.3		0.5		0.3	0.3	0.7
120	tr	tr		18.3	tr			0.6	30.2	30.4	18.7		0.5		0.2	0.2	0.8

^a Chain identification: n, normal (straight chain); br, branched; h, D-(-)-3-hydroxy fatty acid; Δ, unsaturated.^b Equivalent chain length value shown in parentheses.^c tr, Trace (<0.1%).

TABLE 3. Cellular fatty acid profiles of *Actinobacillus* species and *H. aphrophilus*

Organism	Strain	Fatty acid composition (mol%)									
		14br? ^a (13.7) ^b	n14:0 (14.0)	? (14.4)	i15:0 (14.6)	16Δ (15.8)	n16:0 (16.0)	h14:0 (16.1)	18Δ (17.7)	18Δ (17.8)	n18:0 (18.0)
<i>A. seminis</i>	ATCC 15768	1.0	45.2		0.2	20.1	16.5	15.5	2.2		0.3
<i>A. suis</i>	ATCC 15557		15.4	0.4		35.1	28.3	16.0	1.1	0.6	2.2
<i>A. equuli</i>	ATCC 19392		19.0			30.4	27.9	19.4	0.8	0.6	1.9
<i>A. lignieresii</i>	ATCC 19393		25.1	0.6		26.8	30.2	14.5	1.0	0.5	1.3
<i>H. aphrophilus</i>	ATCC 13252		17.6			33.1	32.1	16.3	0.2	0.3	0.5
	ATCC 19415		21.6			31.0	29.1	17.7	0.5		0.2
	NCTC 5906		20.6			28.8	34.0	15.4	0.6		0.6
	NCTC 5907		16.0			24.2	33.7	26.2	tr ^c		tr
	NCTC 5908		10.3			38.7	29.0	20.7	1.3		0.5

^a Chain identification: br, branched; n, normal (straight chain); i, iso-branched; Δ, unsaturated; h, D-(−)-3-hydroxy fatty acid.

^b Equivalent chain length value shown in parentheses.

^c tr, Trace (<0.1%).

FIG. 1. Line drawing of patterns from *Actinobacillus* and *H. aphrophilus* strains: lane (1) *A. suis* ATCC 15557; (2) *A. seminis* ATCC 15768; (3) *A. equuli* ATCC 19392; (4) *A. lignieresii* ATCC 19393; (5-9) *H. aphrophilus* ATCC 13252, ATCC 19415, NCTC 5906, NCTC 5907, NCTC 5908, respectively; (10-20) *A. actinomycetemcomitans* ATCC 29522, ATCC 29523, ATCC 29524, Y4, NCTC 9709, NCTC 9710, 1, 14, 26, 28, 30, respectively.

four *Actinobacillus* species examined. The predominant fatty acids of all 40 strains tested were 14:0, 3-OH 14:0, 16Δ, and 16:0. *A. seminis* (ATCC 15768) had a significantly greater amount of 14:0 than that of the other 39 strains. It should be noted, however, that only one strain each of *A. seminis*, *A. suis*, *A. lignieresii*, and *A. equuli* was tested. The examination of more strains of these four species appears warranted to verify our results.

Three recent reports are available on the cellular fatty acid content of the *Actinobacillus-H.*

aphrophilus group of organisms. Jantzen et al. (7) examined two strains each of *A. actinomycetemcomitans* (NCTC 9709; NCTC 9710) and *H. aphrophilus* (NCTC 5886 [ATCC 19415]; NCTC 5906) and found a predominance of the same four major fatty acids as demonstrated by us. Minor quantitative differences between these authors' results and the present results may be due to the varying cultural and extraction methods utilized. Our results, however, differed qualitatively from those of Braunthal et al. (2). In 15 strains of *A. actinomycetemcomitans* and 5

FIG. 2. Line drawing of patterns from *A. actinomycetemcomitans* strains: lane (1) 34, (2) 37, (3) 39, (4) 45, (5) 55, (6) 56, (7) 64, (8) 68, (9) 73, (10) 74, (11) 76, (12) 78, (13) 79, (14) 81, (15) 88, (16) 93, (17) 97, (18) 112, (19) 117, (20) 120.

strains of *H. aphrophilus*, these authors only detected three major fatty acids (14:0, 16:0, 16:1) and little or no hydroxy fatty acids. Recent findings of Kiley and Holt (8), however, suggested that β -hydroxy fatty acids may be significant constituents of the lipid A moiety of *A. actinomycetemcomitans* lipopolysaccharide.

The soluble protein patterns on polyacrylamide gels stained with Coomassie brilliant blue also suggested an overall similarity among all strains of *A. actinomycetemcomitans*. A marked distinction, however, was found among the protein patterns of *A. actinomycetemcomitans* and both *H. aphrophilus* and other actinobacillus species. These findings indicate that, although the present protein analysis cannot differentiate among strains within the species *A. actinomycetemcomitans*, this method may be useful as a screening method for *A. actinomycetemcomitans* and related species, and may be used to reduce the total analytic time and to verify the identity of strains characterized by conventional tests.

In a taxonomic study of the genus *Haemophilus*, Kilian (9) found a close phenotypic similarity between *A. actinomycetemcomitans* and *H. aphrophilus*, and suggested the possibility of including *A. actinomycetemcomitans* in the genus *Haemophilus*. Even though the available data from fatty acid analyses cannot distinguish *A. actinomycetemcomitans* from *H. aphrophilus*, the soluble protein results indicated that distinct differences exist between these two spe-

cies. Whether the two species should be maintained in different genera or as separate species or whether several species exist within the present species *A. actinomycetemcomitans*, however, awaits further studies, including examinations of the deoxyribonucleic acid relatedness of these organisms.

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