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Fatty Acid Analysis of Microsporidia

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Fatty acid methyl ester (FAME) compositions of three species of microsporidia, *Thelohania* sp., *Vairimorpha necatrix*, and *Nosema algerae* from two insect hosts, were studied. This is the first report on the utility of fatty acid analysis as a parameter for the identification of microsporidia, a group of ubiquitous, intracellular parasites. Three acids, palmitic (16:0), oleic (18:1 ω 9 cis), and two closely eluting acids combined as Summed Feature 6 (18:2 ω 6,9 cis and 18:0 anteiso), compose 60% or more of the total profiles of the three species. The three species were differentiated by a combination of qualitative and quantitative FAME profile features. *Thelohania* sp. and *N. algerae* had myristic (14:0) and gondoic acid (20:1 ω 9 cis). Thus they were qualitatively differentiated from *V. necatrix*, in which 14:0 and 20:1 ω 9 cis were not detected. *Thelohania* sp. had significantly lower amounts of oleic acid than the other two species and significantly higher amounts of 20:1 ω 9 cis than *N. algerae*. FAME composition of *N. algerae* appeared to be influenced by the insect host. Two acids present in *N. algerae* isolated from the mosquito, *Anopheles quadrimaculatus*, were not detected in *N. algerae* recovered from the corn earworm, *Helicoverpa zea*. Quantitatively, host-related differences included higher levels of Summed Feature 6 in *N. algerae* from *H. zea*, compared to *N. algerae* from *A. quadrimaculatus*. The utility of FAME profiles for microsporidian taxonomy will depend on: (1) obtaining profiles from a wider range of microsporidian species, and (2) considering environmental and physiological influences of the host. © 1996 Academic Press, Inc.

KEY WORDS: fatty acids; Microsporidia.

INTRODUCTION

Microsporidia are a large and ubiquitous group of intracellular parasites characterized by unicellular spores containing a polar filament (Sprague *et al.*, 1992). Traditionally, microsporidian taxonomy and clas-

sification have been based on spore morphology, life cycles, and host specificities. Characterization based solely upon morphology can result in misleading classification, because spores of different microsporidian species may appear to be phenotypically identical (Didier *et al.*, 1991) or one species may have several different spore phenotypes, depending on host and life stage (Sweeney *et al.*, 1985). Environmental factors, such as temperature, can affect the expression of different spore phenotypes (Jouvenaz and Lofgren, 1984). Incomplete understanding of the often complex life cycles involving several hosts and spore types hampers experimental transmission of many species in the laboratory.

Molecular techniques, including restriction fragment length polymorphism (RFLP) and sequence alignment of the small 16S rRNA subunit, are being developed for microsporidian species identification and phylogenetic construction (Baker *et al.*, 1995). Additional methodologies, including spore protein profiles (Didier *et al.*, 1991), serological assays (Didier *et al.*, 1991; Oien and Ragsdale, 1992), and flow cytometry (Amigo *et al.*, 1994) have been used to aid in classification, although to a limited extent.

Gas-liquid chromatography of fatty acid methyl esters (FAMEs) is a method used successfully for the identification of bacteria, yeasts, and other fungi (Sasser 1990a,c). Recent studies have extended the utility of fatty acid profile analyses to characterize and distinguish other types of organisms, including vesicular-arbuscular mycorrhizae (Bentivenga and Morton, 1995), viruses (Williams and Thompson, 1995), and fish (Sasser, 1993). Fatty acid distributions may be typical of groups at different taxonomic levels including genera and species (Welch, 1991). This study assessed the application of FAME profiles for the identification and classification of microsporidia represented by three species in three genera.

MATERIALS AND METHODS

Test organisms. Three genera were selected for fatty acid analysis: *Vairimorpha*, *Nosema*, and *Theloha-*

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nia. Vairimorpha necatrix was obtained from J. V. Maddox, Illinois Natural History Survey, and propagated in the corn earworm, *Helicoverpa zea*. *Nosema algerae*, provided by A. H. Undeen (USDA-ARS, Gainesville), was augmented in two insects, *H. zea* and the common malaria mosquito, *Anopheles quadrimaculatus*. *Thelohania* sp. was harvested from field-collected Argentine fire ants, *Solenopsis richteri*, courtesy of R. S. Patterson and J. Briano. The aforementioned species were selected based on availability because many species of microsporidia are very difficult to culture *in vivo* or *in vitro*. Furthermore, *N. algerae* is one of the few species of microsporidia that can be grown easily in different insect hosts, which allowed us to test host influence on FAME profiles.

Spore propagation of *N. algerae* in *H. zea*. Four-day-old *H. zea* larvae were starved individually for 24 hr, then 20 μ l of an aqueous suspension of 1×10^7 spores/ml was added to each. After an additional 24 hr, the larvae were separately placed on a pinto bean diet and maintained at 29°C. Spores were purified from adult *H. zea*.

Spore propagation of *V. necatrix* in *H. zea*. Five-day-old *H. zea* larvae were exposed to 10 μ l of 1×10^6 spores/ml each and raised separately on pinto bean diet at 29°C. Spores were harvested from last instar *H. zea* larvae.

Spore propagation of *N. algerae* in *A. quadrimaculatus*. Approximately 1000 mosquito eggs were hatched in 100 ml water containing 13 mg of a 1:1 mix of dried, powdered liver and brewer's yeast. After 24 hr, this infusion was enriched with 30 mg of alfalfa powder, and the larvae were exposed to 1×10^5 spores of *N. algerae* and raised to adults. Spores were harvested from adult mosquitoes 3–5 days postemergence.

Spore harvest and purification. Last-instar *H. zea* larvae infected with *V. necatrix* were surface-sterilized in 70% ethanol. Fat bodies were removed without lacerating gut tissues, placed into deionized water, ground in a glass tissue grinder, and filtered through cotton. Adult *H. zea* moths infected with *N. algerae* were rinsed in water and, after wing removal, triturated in a Tekmar Tissumizer in deionized water. The resulting suspensions were filtered through a cotton plug in a glass syringe. The crude *V. necatrix* and *N. algerae* spore preparations were further purified by a deionized water wash and differential centrifugation on a continuous Ludox gradient (Undeen and Alger, 1971). Adult infected mosquitoes were immobilized by chilling at -20°C for about 3 min. They then were collected with an aspirator connected to a vacuum pump and homogenized in a small amount of deionized water in a Waring blender. The resulting suspension

was strained through a cotton plug in a syringe to remove large body parts. Further purification was achieved by a deionized water wash and differential centrifugation on a continuous Ludox gradient.

Adult worker ants of *S. richteri* infected with *Thelohania* sp. were triturated in a Tekmar Tissumizer in "ant homogenizing buffer" (0.1% SDS, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The suspension was strained through cotton and centrifuged in deionized water. The pellet was incubated for 10 min at 40°C in 10 μ g/ml proteinase K and $\frac{1}{4}$ vol of "interfacial envelope disruption buffer" (4% SDS, 25 mM EDTA, 50 mM Tris-HCl, pH 7.5), followed by differential centrifugation on a Ludox gradient. All spore preparations were further cleaned by centrifugation on a 100% Percoll gradient, and repeatedly washed in deionized water, prior to fatty acid analysis.

Fatty acid extraction and chromatography. Spore preparations used for fatty acid analysis were examined with a phase contrast microscope prior to extraction to check for bacterial contaminants. Approximately 1×10^9 spores in aqueous suspension were pipetted into a 13 \times 100-mm glass test tube and stored overnight at 4°C to allow the spores to settle. The supernatant was then withdrawn carefully and esterification of fatty acids was accomplished using the method of Miller (1982). Briefly, 1 ml of 15% NaOH in 50% methanol was added to the spore pellet and the tube was capped. Fatty acids were saponified at 100°C for 30 min. Upon cooling, 2 ml of 6 N HCl in 50% MeOH was added, and the tube was recapped and heated at 80°C for 10 min to methylate the fatty acids. Fatty acid methyl esters were solvent-extracted from the aqueous phase with 1.25 ml of hexane:methyl-*tert*-butyl ether (1:1; v/v). The organic phase was washed with 3 ml of 1.2% aqueous NaOH and transferred to a gas chromatograph (GC) vial. Prior to esterification, spore samples of *N. algerae* from the two insect hosts were rinsed with 0.1% SDS to remove externally attached host lipids. Analysis of the SDS solution after rinsing of the spores indicated that no fatty acids, from 9 to 20 carbons in length, were present. To determine the number of microsporidia required for fatty acid analysis, a range of different numbers of spores (3.3×10^8 – 2.1×10^9) was extracted. Although fewer spores could be extracted and derivatized, a sample size of 1×10^9 spores is recommended to provide sufficient area count.

Fatty acid methyl ester extracts were analyzed by the Microbial ID System (MIDI) (Sasser, 1990b), which consists of a computer-linked Hewlett Packard 5890 gas-liquid chromatograph fitted with an Ultra 2 fused silica capillary column (25 m \times 0.2 mm i.d. \times 0.33 μ m film thickness; crosslinked 5% phenyl methyl silicone). Following a $\frac{1}{100}$ split injection of 2 μ l at 170°C, the oven temperature was increased 5°C/min to a final tempera-

ture of 270°C; hydrogen was used as the carrier gas. After flame-ionization, FAME peaks were quantified by a Hewlett Packard 3392 integrator, and expressed as percentages of the total FAME profiles. Data were stored in the MIDI computer for subsequent comparison and statistical analyses. Prior to and between analyses for every 10 samples, a calibration standard mixture consisting of the 12 straight-chain carbon acids from C9 to C20, plus five hydroxy acids, was injected. The resulting retention time and quantitative data served as quality control indicators to ensure good column performance and peak matching by the MIDI system. Periodically, *Xanthomonas maltophilia*, a bacterium whose FAME profile is well characterized, was used as a positive control to ensure reproducibility among different extraction batches.

Representative samples were further characterized by coupled GC–mass spectrometry (GC–MS). Aliquots of the FAME mixtures were analyzed by a Perkin Elmer 8420 GC interfaced with a Finnigan Ion Trap Detector (ITD, Model 6210), with INCOS data collection software and a 80286 computer. The GC–MS was fitted with a 25 m × 0.25 mm i.d. DB-1 fused silica capillary column. The injection of 1 µl was in a splitless mode, followed with a purge flow of helium after 30 sec. The carrier gas helium had a flow rate of 25 cm/sec (Nation *et al.*, 1992). The initial temperature of the column was 60°C; after sample injection, the temperature was programmed to 150°C at 30°C/min, then raised to 220°C at 5°C/min, and held for a total running time of 100 min. Mass spectrometry and comparison of the resulting peaks to the MIDI calibration standard mix and peak library were employed to confirm the identity of the major acids (16:0, 16:1 ω 7 cis, 18:0, 18:1 ω 9 cis, 18:2 ω 6,9 cis) detected in the microsporidia.

Data analysis. Fatty acid methyl esters of the samples were named by comparing their retention times to those of a known standard (a mixture of straight-chain saturated fatty acids from 9 to 20 carbons in length including five hydroxy acids). Retention time data from the injected calibration mixture can be converted to Equivalent Carbon Length (ECL) data for fatty acid naming. Thus, the ECL value for each compound to be analyzed is computed, and the compounds are named based on comparisons to the standards as well as acids stored in the MIDI peak library (142 peaks total) (Sasser, 1990b).

A library was created from the resulting fatty acids, and library entries were analyzed with Principal Component Analysis. Sources of variability and clustering among the profiles were graphically assessed by plotting the percentages of the three major fatty acids on a 3-D graph. Statistical analysis of the fatty acid data was performed with an analysis of variance (ANOVA) followed by a Tukey's mean separation test to compare

the means of each fatty acid among the microsporidian species tested (SAS Institute, 1989).

RESULTS AND DISCUSSION

Fatty acid methyl ester profile analyses of microsporidia have not been reported prior to this study even though FAME profiles of other protozoa such as *Tetrahymena pyriformis* (Chu *et al.*, 1972) and *Trypanosoma cruzi* (Leon *et al.*, 1989) have been determined. The fatty acid profiles of those protozoa and the microsporidia are similar but there are quantitative as well as qualitative differences. *Trypanosoma cruzi*, for example, has linolenic acid (18:3) but the microsporidia do not. The three microsporidian species analyzed in this study could be differentiated by a combination of qualitative and quantitative FAME profile characteristics (Table 1, Fig. 1).

Three acids, palmitic (16:0), oleic (18:1 ω 9 cis), and two closely eluting peaks denoted as Summed Feature 6 (18:2 ω 6,9 cis/18:0 anteiso), composed 60% or more of the total FAME profiles of *V. necatrix*, *N. algerae*, and *Thelohania* sp. Palmitic acid is ubiquitous and present in all organisms (Myron Sasser, personal communication). Oleic acid is also widely distributed and present for example in many bacterial human pathogens, phytopathogens, vesicular–arbuscular mycorrhizae (Welch, 1991; Sasser, 1990a; Bentivenga and Morton, 1995), and *T. cruzi* (Leon *et al.*, 1989). A comprehensive list of fatty acids characteristic of a wide variety of organisms is presented elsewhere (Kerwin, 1994). Myristic (14:0) and gondoic acid (20:1 ω 9 cis) were present at low levels in all of the *N. algerae* and *Thelohania* sp. samples; however, these acids were not detected in *V. necatrix* (Table 1). Quantitative differences included significantly lower levels of palmitic acid (16:0) in *Thelohania* sp. than the other two species ($P < F = 0.0001$; $df = 3,22$; $\alpha = 0.05$) and significantly higher levels of myristic (14:0) and gondoic acid (20:1 ω 9 cis) in *Thelohania* sp. than *N. algerae* ($P < F = 0.0001$; $df = 3,22$; $\alpha = 0.05$). Oleic acid (18:1 ω 9 cis) was present at significantly higher amounts in *V. necatrix* than in *N. algerae* ($P < F = 0.0001$; $df = 3,22$; $\alpha = 0.05$).

Three fatty acids not present in the MIDI Microbial Identification System (MIS) peak library index were found in several *Thelohania* sp. samples (Table 1). These acids could be typical of developing spores (physiological age differences of spores), or representative of the organism. Similar instances of unnamed fatty acids have recently been reported in studies on vesicular–arbuscular mycorrhizae (Nancy Hodge, personal communication). Together, the three acids composed $16.3 \pm 9.8\%$ of the total FAME profiles in *Thelohania* sp. samples. The acids eluted from the MIDI system's capillary column at 9.486, 11.915, and 12.531 min, and had ECL of 15.565, 17.014, and 17.368, respectively. Structural determination of the unnamed acids would

TABLE 1
Percentage of Fatty Acids in Three Microsporidian Species from One Aquatic and Two Terrestrial Insect Hosts

Fatty acid ^a	<i>Nosema algerae</i>		<i>Vairimorpha</i>	<i>Thelohania</i> sp.
	<i>A. quadrimaculatus</i> (n = 7)	<i>H. zea</i> (n = 7)	<i>necatrix</i> <i>H. zea</i> (n = 5)	<i>S. richteri</i> (n = 7)
14:0	2.8 ± 0.2 ^b B ^c	2.2 ± 0.1 C	0.0 D	4.7 ± 0.2 A
16:1 $\bar{\omega}$ 7cis	11.4 ± 0.8 A	2.0 ± 0.1 C	1.4 ± 0.2 C	4.2 ± 0.7 B
16:0	34.5 ± 0.6 A	34.1 ± 0.5 A	31.2 ± 1.5 A	22.7 ± 1.0 B
Summed Feature 6 ^d	13.4 ± 0.8 B	25.4 ± 0.8 A	28.4 ± 2.2 A	17.4 ± 1.3 B
18:1 $\bar{\omega}$ 9cis	16.5 ± 0.3 C	20.5 ± 1.8 BC	29.6 ± 2.9 A	26.0 ± 2.1 AB
Summed Feature 7 ^e	6.3 ± 0.5 A	0.0 B	0.0 B	0.2 ± 0.2 B
18:0	4.7 ± 0.2 BC	9.4 ± 0.2 A	6.5 ± 0.9 B	4.1 ± 0.6 C
20:4 $\bar{\omega}$ 6,9,12,15cis	4.1 ± 0.3 A	0.0 B	0.0 B	2.7 ± 0.6 A
20:1 $\bar{\omega}$ 9cis	1.8 ± 0.4 BC	2.6 ± 0.1 B	0.0 C	6.3 ± 0.8 A

^a Number of carbon atoms in fatty acid:number of double bonds per acid.

^b Standard error of the mean.

^c Arithmetic means in a row followed by a different letter are significantly different from each other.

^d Summed Feature 6 identified as 18:2 $\bar{\omega}$ 6,9cis/18:0 anteiso by the MIDI peak library.

^e Summed Feature 7 identified as 18:1 $\bar{\omega}$ 7cis/ $\bar{\omega}$ 9trans/ $\bar{\omega}$ 12trans by the MIDI peak library.

be best confirmed using ancillary GC-MS and nuclear magnetic resonance techniques.

Host influence on FAME profiles of the spores was tested by producing *N. algerae* in *H. zea* and *A. quadrimaculatus*. Three closely eluting 18:1 cis-trans isomers,

combined as Summed Feature 7 (18:1 $\bar{\omega}$ 7 cis/18:1 $\bar{\omega}$ 9 trans/18:1 $\bar{\omega}$ 12 trans), and arachidonic acid (20:4 $\bar{\omega}$ 6,9,12,15 cis) were detected in *N. algerae* isolated from mosquito, at 6.3 and 4.1%, respectively (Table 1). Neither acid, however, was present in *N. algerae* from

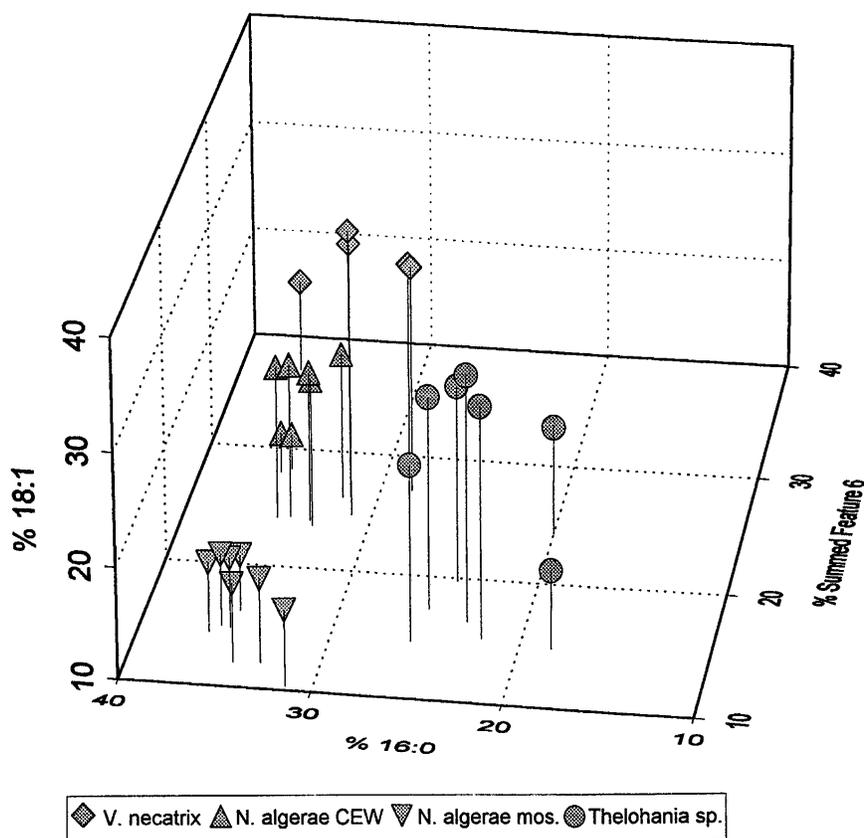


FIG. 1. Major fatty acids of three species of microsporidia. Measurements of variability and clustering among the profiles were portrayed by plotting the percentages of the three major fatty acids on a 3-D graph. CEW, corn earworm; mos., mosquito.

corn earworm. Furthermore, the percentages of three other acids varied according to host. In *N. algerae* from corn earworm, levels of 16:1 \bar{w} 7 cis, 18:2 \bar{w} 6,9 cis/18:0 anteiso (Summed Feature 6), and 18:0 (stearic acid) were 2.0, 25.4, and 9.4%, respectively. Levels of those acids in *N. algerae* isolated from mosquito were significantly different with 11.4, 13.4, and 4.7%, respectively ($P < F = 0.0001$; $df = 3,22$; $\alpha = 0.05$). A more rigorous analysis of the polyunsaturated fatty acids would require extended running times and appropriate standards on the GC MIDI system (Sasser, 1993) and a polar column (Suzuki *et al.*, 1993) with appropriate standards on the GC-MS system.

A variety of environmental factors, including age, culture medium, pH, and growth temperature have been shown to affect FAME profiles of bacteria (Lechevalier, 1989) and fungi (Marumo and Aoki, 1990). In bacteria, stability of the FA composition is achieved through growth under standardized conditions *in vitro*. Fatty acid stability is optimal in bacteria growing at the late log or early stationary growth phase (Sasser, 1990b). Even though environmental factors may influence FAME compositions, spores of several species of glomalean fungi yielded reproducible FAME profiles despite being grown in association with a host plant and with contaminating microorganisms present (Graham *et al.*, 1995). Another study with glomalean fungi also demonstrated that host physiology was not a significant factor in causing modifications to composition or abundance of fatty acids (Bentivenga and Morton, 1995). The present study showed, however, that *N. algerae* had a qualitatively and quantitatively distinct profile depending on the host (Table 1, Fig. 1). Glomalean endomycorrhizal fungi are similar to many microsporidia in that they cannot be cultured without their hosts. Taxonomy of glomalean fungi is based on spore morphology. Similar to microsporidia, assessment of diversity using only morphological characters is difficult because of inadequately defined characters, and ambiguous distinction between morphologically similar species (Morton, 1993).

Principal Component Analysis of the three major acids found in the microsporidia graphically portrayed the clustering of the three species (Fig. 1). *Thelethania* sp. was separated from the other species because of its low levels of 16:0. *Nosema algerae* and *V. necatrix* both had high levels of 16:0, but *V. necatrix* also had high amounts of 18:1 \bar{w} 9 cis, which distinguishes it from *N. algerae*. Host insect affected the clustering of *N. algerae*. Lower levels of Summed Feature 6 were detected in *N. algerae* from mosquito than *N. algerae* from corn earworm. Variability in the FAME profiles of the individual samples was lowest in *N. algerae* from mosquito (Table 1, Fig. 1).

Host influence on FAME profiles together with the requirement of a large sample size of roughly 1×10^9

spores for fatty acid extraction may render fatty acid analysis unsuitable as a tool for microsporidian identification. The fatty acid extraction process could be scaled down, however, to accommodate for smaller sample sizes (Welch, 1991). Furthermore, *in vivo* and *in vitro* culture techniques of microsporidia could be developed and refined to grow microsporidia under standardized conditions and in sufficient quantity. More studies with additional microsporidian species from a variety of environmental conditions (different animal host) are needed to determine fatty acids characteristic for the microsporidia and to assess qualitative and quantitative aspects of fatty acid analysis as a discriminant tool in identification. The compilation of microsporidian FAME profiles will enable statistical comparisons using the MIDI pattern recognition software. By comparing profiles of well-characterized reference microsporidia, the utility of FAME analysis as a taxonomic tool for the identification of microsporidia could be evaluated.

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