***Entomoplasma freundtii* sp. nov., a new species from a green tiger beetle (Coleoptera: Cicindelidae)**

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A mollicute (strain BARC 318^T) isolated from gut tissue of a green tiger beetle (Coleoptera: Cicindelidae) was found by dark-field microscopy to consist of non-helical, non-motile, pleomorphic coccoid forms of various sizes. In ultrastructural studies, individual cells varied in diameter from 300 to 1200 nm, were surrounded by a cytoplasmic membrane and showed no evidence of cell wall. The organisms were readily filterable through membrane filters with mean pore diameters of 450 and 300 nm, with unusually large numbers of organisms filterable through 200 nm pore membrane filters. Growth occurred over a temperature range of 15–32 °C with optimum growth at 30 °C. The organism fermented glucose and hydrolysed arginine but did not hydrolyse urea. Strain BARC 318^T was insensitive to 500 U penicillin ml⁻¹ and required serum or cholesterol for growth. It was serologically distinct from all currently described sterol-requiring, fermentative *Mycoplasma* species and from 12 non-sterol-requiring *Mesoplasma* species, 13 non-sterol-requiring *Acholeplasma* species and 5 previously described sterol-requiring *Entomoplasma* species. Strain BARC 318^T was shown to have a G+C content of 34 mol% and a genome size of 870 kbp. The 16S rDNA sequence of strain BARC 318^T was compared to 16S rDNA sequences of several other *Entomoplasma* species and to other representative species of the genera *Spiroplasma* and *Mycoplasma*, and to other members of the class *Mollicutes*. These comparisons indicated that strain BARC 318^T had close phylogenetic relationships to other *Entomoplasma* species. On the basis of these findings and other similarities in morphology, growth and temperature requirements and genomic features, the organism was assigned to the genus *Entomoplasma*. Strain BARC 318^T (ATCC 51999^T) is designated the type strain of *Entomoplasma freundtii* sp. nov.

Keywords: *Entomoplasma freundtii* sp. nov., mollicute, green tiger beetle

INTRODUCTION

The reported occurrence of a variety of wall-less prokaryotes (class *Mollicutes*) in numerous insect and plant hosts has posed a number of taxonomic problems (27). In earlier studies on mollicutes in such habitats, helical organisms were assigned to the genus *Spiroplasma* (36, 46), while non-helical, wall-less organisms

were assigned to *Mycoplasma* or *Acholeplasma* (18, 33–35, 39, 45), depending upon their sterol growth requirement. However, phylogenetic analysis of mollicutes from a variety of habitats (41) and additional genetic and physiological information (genome size, codon usage, glucose transport enzymes) (5, 6, 19, 20) from some non-helical mollicutes from insect/plant habitats clearly define many of these organisms as distinct from classic *Acholeplasma* or *Mycoplasma* species. Although a few of the organisms have proved to be true *Acholeplasmas* (38), most of them belong to

The GenBank accession number for the sequence reported in this paper is AF036954.

a distinct lineage that has arisen from the spiroplasmas (41). These findings resulted in a proposed revision of the taxonomy of the class *Mollicutes* (31) in which insect- and plant-derived mollicutes were combined in a new order *Entomoplasmatales*, with helical mollicutes (family *Spiroplasmataceae*, genus *Spiroplasma*) and non-helical mollicutes (*Entomoplasmataceae*) being accorded separate status.

All members of the *Entomoplasmataceae* of insect or plant origin have been found to have an optimum growth temperature of 30 °C and a genome size between 790 and 1140 kbp. Organisms with a clear growth requirement for cholesterol were assigned to the genus *Entomoplasma*, which now contains five recognized species (31, 32). Non-helical mollicutes without a cholesterol requirement but with a requirement for small fatty acid supplements were placed in the genus *Mesoplasma* (31, 37). Thirteen species in this genus are presently recognized (32).

A filterable, non-helical and non-motile mollicute, designated strain BARC 318^T, was isolated in June 1987 from the gut contents of a green tiger beetle (Coleoptera: Cicindelidae). The insect was collected in an upland deciduous woodland area with sandy soil at the Beltsville Agricultural Research Center, MD, USA. The non-helical morphology and non-motile activity of the organism excluded assignment of the organism to the genus *Spiroplasma*, so further efforts were made to determine whether the organism might belong to other genera within the class *Mollicutes* (*Acholeplasma*, *Mesoplasma* or *Entomoplasma*). These comparative studies eventually established that the organism was similar in a number of critical biological properties to non-helical mollicutes assigned to the genus *Entomoplasma*, although it was serologically distinct from the five *Entomoplasma* species previously described. In this report we provide the specific characteristics of strain BARC 318^T in fulfilment of the minimum standards for new species descriptions within the class *Mollicutes* (14) and propose the name *Entomoplasma freundtii* sp. nov.

METHODS

Organisms. Gut contents from a green tiger beetle (Cicindelidae) were transferred to M1D medium (42) containing 500 U penicillin ml⁻¹ and the suspension was passed through

a sterile 450 nm membrane filter. The filtrate was incubated at 30 °C and after 7 d the broth culture displayed an acidic shift in pH. Dark-field examination of the culture revealed numerous non-helical, non-motile organisms. Strain BARC 318^T was then purified by conventional filtration cloning techniques (28). Type strains of five previously recognized species of the genus *Entomoplasma* (32) were also employed in comparative studies with strain BARC 318^T (Table 1).

Culture medium and cultivation techniques. SP-4 (42) or M1D culture media were used for most characterization work. In biochemical and sterol test procedures, strain BARC 318^T was adapted to grow in a reduced serum medium containing 1% bovine serum fraction (42). Agar formulations of SP-4 medium were prepared by adding Noble agar (Difco) to a final concentration of 0.8%. Temperature requirements for growth, including optimum temperature and temperature range, were assessed as previously described (16). Following the results of these tests, most characterization studies were performed at 30 °C with agar cultures incubated anaerobically in the Gaspak system (BBL Microbiology Systems).

Morphological studies. Cells of strain BARC 318^T in exponential phase were examined by dark-field microscopy at a magnification of ×1250. The ultrastructure of this strain, as determined by electron microscopic examination of sectioned cells, was determined as previously reported (44).

Sterol requirement. Sterol requirement for growth of strain BARC 318^T was assayed in a test measuring the ability of the organism to sustain growth in a serum-free medium, in serum-free medium with a fatty acid supplement (0.04% polyoxyethylene sorbitan) or in a medium containing 15% foetal bovine serum (23, 30).

Tests for biological and biochemical properties. Procedures for assessing glucose fermentation and hydrolysis of arginine or urea have been described (1). Filtration characteristics of the organism following passage of SP-4 broth cultures through membrane filters of various porosities (450, 300 and 200 nm) were measured by techniques reported previously (28).

Serological tests. Antiserum to strain BARC 318^T was produced in rabbits as previously described (25, 29). The agar plate growth inhibition test (9) and a metabolism inhibition test in broth (26) were used to compare the response of strain BARC 318^T to hyperimmune antisera to various *Entomoplasma* species. In addition, strain BARC 318^T was also tested using the growth inhibition procedure against 74 antisera to type strains of all currently established fermentative or non-arginine-hydrolysing *Mycoplasma* species, to 13 antisera to current *Acholeplasma* species and to 12

Table 1. Reciprocal metabolism inhibition tests with BARC 318 and established *Entomoplasma* species

Antigen	Reciprocal metabolism inhibition titre of hyperimmune sera against strain:					
	ELCN-1 ^T	PIPN-2 ^T	PIMN-1 ^T	M1 ^T	PYAN-1 ^T	BARC 318 ^T
<i>Entomoplasma ellychniae</i> ELCN-1 ^T	> 20000	64	< 16	< 16	< 16	< 16
<i>Entomoplasma lucivorax</i> PIPN-2 ^T	< 16	> 20000	< 16	< 16	< 16	< 16
<i>Entomoplasma luminosum</i> PIMN-1 ^T	< 16	< 16	1280	< 16	< 16	< 16
<i>Entomoplasma melaleuca</i> M1 ^T	< 16	512	64	> 20000	< 16	< 16
<i>Entomoplasma somnilux</i> PYAN-1 ^T	< 16	< 16	< 16	< 16	> 20000	< 16
Strain BARC 318 ^T	< 16	< 16	< 16	< 16	< 16	> 20000

Table 2. Mollicutes and other bacterial species used in this study

Species	Strain	Nucleotide sequence accession no.	Reference
<i>Acholeplasma laidlawii</i>	PG8 ^T	U14905	2
<i>Anaeroplasma abactoclasticum</i>	6-1 ^T	M25050	41
<i>Asteroleplasma anaerobium</i>	161 ^T	M22351	41
<i>Bacillus subtilis</i>	168	M10606	13
<i>Clostridium innocuum</i>	B-3 ^T	M23732	41
<i>Clostridium ramosum</i>	113-I ^T	M23731	41
<i>Entomoplasma ellychniae</i>	ELCN-1 ^T	M24292	41
<i>Entomoplasma freundtii</i>	BARC 318 ^T	AF036954	This study
<i>Entomoplasma melaleuca</i>	M1 ^T	M24478	41
<i>Escherichia coli</i>	K-12	J01695	4
<i>Mesoplasma entomophilum</i>	TAC ^T	M23931	41
<i>Mesoplasma lactucae</i>	831-C4 ^T	M24479	41
<i>Mycoplasma capricolum</i> subsp. <i>capricolum</i>	California Kid ^T	X00921	15
<i>Mycoplasma hominis</i>	PG21 ^T	M24473	41
<i>Mycoplasma mycoides</i> subsp. <i>mycoides</i>	PG1 ^T	M23943	41
<i>Mycoplasma pneumoniae</i>	FH ^T	M29061	41
<i>Mycoplasma putrefaciens</i>	KS-1 ^T	M23938	41
Phytoplasma apple proliferation	AT	X68375	24
Phytoplasma stolbur	STOL	X76427	24
<i>Spiroplasma apis</i>	B-31 ^T	M23937	41
<i>Spiroplasma citri</i>	Maroc/R8A2 ^T	M23942	41
<i>Spiroplasma clarkii</i>	CN-5 ^T	M24474	41
<i>Spiroplasma diabroticae</i>	DU-1 ^T	M24482	41
<i>Spiroplasma gladiatoris</i>	TG-1 ^T	M24475	41
<i>Spiroplasma ixodetis</i>	Y32 ^T	M24477	41
<i>Spiroplasma mirum</i>	SMCA ^T	M24662	41
<i>Spiroplasma monobiae</i>	MQ-1 ^T	M24481	41
<i>Spiroplasma taiwanense</i>	CT-1 ^T	M24476	41
<i>Spiroplasma</i> sp. (<i>Drosophila</i>)	DW-1	M24483	41
<i>Streptococcus pneumoniae</i>	NCTC 7465 ^T	X58312	3

antisera to established *Mesoplasma* species (32). These sera originated from a reference collection at the National Institute of Allergy and Infectious Diseases Laboratory, Frederick, MD, USA.

Genomic analysis. The DNA base composition (G+C content) of strain BARC 318^T was determined by buoyant density and melting temperature using procedures described previously (7, 8). *Spiroplasma citri* DNA (G+C = 26 mol%) was used as the control for this procedure. The genome size of strain BARC 318^T was determined by PFGE, using techniques outlined previously (5, 6).

Sequence determination and phylogenetic analysis. Nucleic acid was extracted from a BARC 318^T cell pellet using a Chelex resin/boil protocol (40). Two microlitres of a tenfold dilution of the Chelex extraction was added to a 100 µl PCR reaction containing 50 µl Microseq 16S rRNA Gene Kit PCR Master Mix (Applied Biosystems) and 48 µl water. The PCR product generated was 1540 bp in length. Prior to cycle sequencing, the PCR products were purified using a Microcon 100 column (Amicon). The product (100 µl) was added to the top of the column with 400 µl 10 mM Tris/HCl and

0.1 mM EDTA, pH 8.0. The column was spun at 3000 r.p.m. in an Eppendorf 5415C benchtop centrifuge for 15 min. The column was then inverted and spun in the centrifuge again at 3000 r.p.m. for 2 min. The purified product was then brought up to a volume of 50 µl with 10 mM Tris/HCl and 0.1 mM EDTA, pH 8.0.

For cycle sequencing, the Microseq 16S rRNA Gene Kit Sequencing Module (Applied Biosystems) was used according to the manufacturer's instructions. The reactions were cycled using a Gene Amp 9600 Thermal Cycler (Perkin-Elmer). The cycling profile consisted of a 96 °C/10 s denaturation step, followed by a 58 °C/1 min annealing/extension step for a total of 30 cycles. After cycle sequencing, excess dye terminators were removed using Centri-Sept Spin Columns (Princeton Separations) according to the manufacturer's instructions. The reactions were then dried down in a Speed Vac (Savant Instruments) and resuspended in 4 µl sequencing gel loading buffer, which consisted of Blue Dextran (50 mM), EDTA (50 mg ml⁻¹) and deionized formamide (1:5, v/v). The reactions were then loaded onto a 377 DNA Sequencer (Applied Biosystems). Two micro-

litres of the resuspended reaction were loaded into each lane of the 36-lane gel. A 36 cm, 5% Long Ranger polyacrylamide gel (FMC Bioproducts) was used. The samples were electrophoresed at 1650 V and 52 °C for 7 h.

The collected sequences were then analysed using the software programs Sequencing Analysis and Factura (Applied Biosystems). The analysed sequences were then assembled and edited using Auto Assembler (Applied Biosystems) and a consensus sequence was generated. Further comparative analysis was performed using fastDNAm1 (21) and the data and services provided by the Ribosomal Database Project (17). A phylogenetic tree was constructed using TREEVIEW (22). The accession numbers of sequences used in the phylogenetic analysis in this study are listed in Table 2.

RESULTS AND DISCUSSION

Cultural and morphological properties

Strain BARC 318^T grew well in either SP-4 or M1D medium and on a solid medium prepared from these formulations. The strain also grew in the Edward formulation of conventional mycoplasma medium (42) containing 15–20% horse serum, or in conventional mycoplasma medium (42) containing 1% bovine serum fraction. Growth occurred in broth medium containing 500 U penicillin ml⁻¹. The optimum temperature for growth was 30 °C, with growth occurring over a range of 15–32 °C. No growth was recorded during a 3-week period in broth medium incubated at 37 °C. Agar colonies of strain BARC 318^T (Fig. 1) exhibited a granular-type growth, frequently with numerous small satellite colonies and without the typical fried-egg morphology observed with many other mollicutes.

Dark-field microscopy of exponential-phase cultures

Fig. 1. Colonies of strain BARC 318^T on 0.8% SP-4 agar medium after 9 d incubation at 30 °C in an anaerobic environment. Bar, 100 μm.

Fig. 2. Electron photomicrograph of a thin section of BARC 318^T cells showing pleomorphic coccoid cells. Arrows indicate single cytoplasmic membrane. Bar, 200 nm.

of strain BARC 318^T showed non-helical, non-motile, very pleomorphic forms, mostly coccoid in shape. Sectioned cells observed by electron microscopy also demonstrated a variety of pleomorphic coccoid forms with no evidence of cell wall. Representative cells had a diameter in the range 300–1200 nm with individual cells surrounded only by a cytoplasmic unit membrane (Fig. 2).

Sterol requirement

In tests to measure the sustained growth of strain BARC 318^T through 23 serial tenfold passages in three different media, continued growth occurred only in the medium containing 15% foetal bovine serum. The organism was not able to sustain growth beyond the first tenfold dilution in serum-free medium or in medium containing a 0.04% polyoxyethylene sorbitan (Tween 80) supplement, indicating a growth requirement for cholesterol or serum (23, 30).

Biochemical and biological properties

Strain BARC 318^T rapidly fermented glucose with a shift in medium pH to the acid range within 24 h at both room temperature and 30 °C. In broth containing

Fig. 3. Dendrogram showing the phylogenetic position of strain BARC 318^T in relation to other representatives of the class *Mollicutes* and to other bacteria, based on 16S rDNA. Scale bar, 10 substitutions per 100 nt.

arginine but no glucose, the organism induced a slight initial decrease in pH but later produced a rapid change in medium pH towards the alkaline range, indicating the hydrolysis of arginine and concomitant production of ammonia. Tests for urea hydrolysis were negative. Passage of broth cultures of strain BARC 318^T through 450 or 300 nm membrane filters did not reduce the viable cell titre [$\geq 10^9$ colour-changing units (c.c.u.) ml⁻¹]. The titre of a broth culture filtrate obtained after passage through a membrane with a mean pore diameter of 200 nm showed a viable cell population reduced by a factor of about 10 (10^8 c.c.u. ml⁻¹).

Serological tests

Preliminary growth inhibition tests with strain BARC 318^T against 74 antisera to fermentative (or non-arginine-hydrolysing) *Mycoplasma* species, 13 *Acholeplasma* species, 12 *Mesoplasma* species and 5 *Entomoplasma* species indicated the absence of any serological relationship. Antiserum prepared to strain BARC 318^T produced a homologous growth inhibition zone of 5 mm. Since the morphology and growth requirements indicated a closer relationship to *Entomoplasma* species, more extensive serological testing was carried out with antisera to established species in

Table 3. Genomic and growth temperature properties of established *Entomoplasma* species

The optimum growth temperature for all strains was 30 °C. None of the strains grew at 37 °C.

Species	G + C (mol %)	Genome size (kbp)	Growth temperature range (°C)
<i>Entomoplasma ellychniae</i> ELCN-1 ^T	27.5	912	18–32
<i>Entomoplasma lucivorax</i> PIPN-2 ^T	27.4	1188	18–32
<i>Entomoplasma luminosum</i> PIMN-1 ^T	28.8	889	10–32
<i>Entomoplasma melaleucaae</i> M1 ^T	27.0	898	10–30
<i>Entomoplasma somnilux</i> PYAN-1 ^T	27.4	822	10–32
BARC 318 ^T	34.0	870	10–32

this genus. Reciprocal metabolism inhibition tests were performed with antigens and antisera to five established *Entomoplasma* species and to strain BARC 318^T antigen and its homologous antiserum (Table 1). These additional serological tests again confirmed the lack of any serological relationship between strain BARC 318^T and established *Entomoplasma* species. These findings indicate that antisera to both *Mesoplasma* and *Entomoplasma* species should be part of any identification scheme for identifying mollicutes from insect hosts (43).

Genomic characterization

The G + C content of strain BARC 318^T was found to be 34 mol %. The genome size was 870 kbp by PFGE.

Phylogenetic analysis

The first analysis using the Similarity Rank tool from the Ribosomal Database Project indicated that the BARC 318^T 16S rDNA sequence was not identical to any known and deposited sequence. However, it was most closely related to the 16S rDNA sequence of organisms considered as the '*Mycoplasma mycoides* cluster' (including *Mycoplasma capricolum* subsp. *capricolum* and *Mycoplasma putrefaciens*) within the *Spiroplasma* group, as defined by Weisburg and colleagues (41). Further phylogenetic analysis was performed using a subset of the database including all the members of the *Spiroplasma* group sequenced so far, as well as representatives of other phylogenetic groups within the class *Mollicutes* and their walled relatives. Although unrooted, the tree was constructed using *Escherichia coli* as an outgroup (Fig. 3). Strain BARC 318^T clearly belongs to the *Spiroplasma* phylogenetic group and more precisely within this group to members of the *Mycoplasma mycoides* cluster and to organisms assigned to the genera *Entomoplasma* and *Mesoplasma*. Indeed, this overall group contains all the non-helical members of the *Spiroplasma* group sequenced so far. Thus, in the present classification of

the class *Mollicutes* (31), the phylogenetic position of BARC 318^T is consistent with its assignment to the genus *Entomoplasma*.

This reconstruction, and previously constructed trees of the class *Mollicutes* (41), suggests that phylogenetic distances between some mesoplasmas and some entomoplasmas are very small. Furthermore, *Mesoplasma* and *Entomoplasma* may not be monophyletic. At the time that these genera were proposed (31), sterol requirements of mollicutes were considered to be appropriate characteristics for separation of higher mollicute taxa. Since then it has been discovered that members of the genus *Spiroplasma* defined originally as sterol-requiring, may or may not require sterols (23). Although molecular phylogenetic studies of this genus are incomplete, sterol utilization does not appear to be correlated with phylogeny. It would be useful in future to investigate phylogenetic relationships within the genus *Spiroplasma* using protein-encoding genes to relate phenotypic characteristics. Very few molecular phylogenetic determinations have been made on *Mesoplasma* or *Entomoplasma* species. The confusing profile obtained so far, plus the proximity of the members of the order *Entomoplasmatales* to the *Mycoplasma mycoides* cluster, makes further phylogenetic study of the *Entomoplasmatales* imperative. It is possible that such studies, when completed, could indicate that species within the family *Entomoplasmataceae* should be classified in a single genus.

Habitat

The occurrence of strain BARC 318^T in the insect gut is consistent with what is presently known of the habitat of other *Entomoplasma* species. All five currently described *Entomoplasma* species were isolated from the gut or haemolymph of various firefly beetles (*Entomoplasma somnilux*, *Entomoplasma luminosum*, *Entomoplasma lucivorax* and *Entomoplasma ellychniae*), or from an anthophorine bee (*Entomoplasma melaleucaae*) (34, 35, 45). It is also quite likely that BARC 318^T and related strains occur on plant surfaces, since some entomoplasmas (e.g. *Entomoplasma*

melaleucae) were isolated from flowers of several tropical plants (*Melaleuca quinquenervia*, *Melaleuca decora*) and flowers of the silk oak (*Grevillea robusta*) (35).

The properties of strain BARC 318^T described herein fulfill recently revised criteria (14) for new species descriptions in the class *Mollicutes*. Properties mandating assignment to this class include absence of a cell wall, filterability and penicillin resistance. The non-helical morphology of strain BARC 318^T, the optimum growth temperature of 30 °C, the inability to hydrolyse urea and a genome size of 870 kbp place the organism within the order *Entomoplasmatales*, family *Entomoplasmataceae* (Table 3) (31). A sterol requirement indicates the organism belongs in the genus *Entomoplasma* (31). Finally, the lack of a serological relationship of strain BARC 318^T to the type strains of previously established *Entomoplasma* species demonstrates its stature as a distinct species. Accordingly, we propose the name *Entomoplasma freundtii* sp. nov. for this organism, in consideration of the major contributions and pioneering efforts of Eyvind Freundt in the early characterization and classification of the mollicutes (10–12).

Description of *Entomoplasma freundtii* sp. nov.

Entomoplasma freundtii (fr.eun.d'ti.i. M.L. gen. n. *freundtii* named after Eyvind Freundt, a Danish pioneer in the taxonomy and classification of mollicutes).

Cells predominately coccoid in shape, varying from 300 to 1200 nm in diameter. Cells devoid of cell wall and surrounded only by cytoplasmic membrane. Non-motile. Colonies on solid medium with 0.8% agar are granular and frequently exhibit multiple satellite forms. Chemo-organotroph. Acid produced from glucose. Hydrolyses arginine, but not urea. Serum or sterol required for sustained growth. Growth temperature range, 10–32 °C; optimum growth temperature, 30 °C. Serologically distinct from all other established *Entomoplasma* species, as well as currently described glucose-fermenting (arginine-non-hydrolysing) *Mycoplasma*, *Acholeplasma* and *Mesoplasma* species. Isolated from gut contents of a green tiger beetle (Coleoptera: Cicindelidae). Evidence of insect pathogenicity not determined. G+C content, 34 ± 1 mol%; genome size, 870 kbp. Type strain is BARC 318^T (= ATCC 51999^T).

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