NOTE

***Fusobacterium equinum* sp. nov., from the oral cavity of horses**Matthias Dorsch,^{1†} Daria N. Love^{2†} and Graham D. Bailey³¹ Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia² Department of Veterinary Anatomy and Pathology, University of Sydney, NSW 2006, Australia³ NSW Agriculture, Regional Veterinary Laboratory, Orange Agriculture Institute, Forest Road, Orange, NSW 2800, Australia

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Two strains of Gram-negative, anaerobic, non-sporulating rod that were isolated from the normal oral cavity and oral-associated disease from horses and which phenotypically resembled *Fusobacterium necrophorum* were characterized by sequencing of the 16S rRNA gene, phylogenetic analysis, DNA–DNA hybridization and phenotypic characterization. The results placed the novel strains as distinct members of the genus *Fusobacterium*. The novel species *Fusobacterium equinum* sp. nov. is proposed, with strain VPB 4027^T (= NCTC 13176^T = JCM 11174^T) as the type strain.

Keywords: *Fusobacterium*, horses, oral cavity, oral-associated diseases, non-sporulating anaerobes

Members of the genus *Fusobacterium* are amongst a mixed facultative and anaerobic bacterial flora that may be isolated from the normal oral cavity as well as from oral-associated diseases of horses (Bailey & Love, 1991) and from a variety of other soft-tissue infections of humans and other animals. Respiratory tract infections of horses, including necrotizing pneumonia and pleurisy, are commonly associated with transportation. *Fusobacterium* species are a significant part of the anaerobic flora under these conditions (Racklyeft & Love, 1999, 2000).

During a study of the normal flora and oral-associated diseases of horses, a number of isolates resembling *Fusobacterium necrophorum* and feline strains of *Filifactor alocis* (formerly *Fusobacterium alocis*) (Love *et al.*, 1987) were characterized both genetically and phenotypically (Bailey & Love, 1991). These findings suggested that the isolates belonged to a distinct genetic cluster, unlike any yet characterized. Since that time, considerable changes to the taxonomy of members of the genus have taken place, with two species being reassigned to Gram-positive genera on the grounds of phylogenetic analysis of the 16S rRNA gene (Collins *et al.*, 1994; Jalava & Eerola, 1999). It is clear that such analyses are important in the des-

ignation and characterization of organisms that, in the past, were assigned on the basis of phenotypic characteristics including Gram staining and metabolic fatty acid analyses.

In this paper, we describe the results of sequencing of the 16S rRNA genes of two isolates amongst a group of Gram-negative non-sporulating rods that phenotypically resemble *Fusobacterium necrophorum* and show that, while they are most closely related to *Fusobacterium necrophorum*, they are distinct from it. Nevertheless, our analysis suggests that they remain within the currently defined *Fusobacterium* cluster. We therefore propose a new species, *Fusobacterium equinum* sp. nov., a description of which is given.

All strains used in this study are shown in Tables 1 and 2 and Fig. 1. *Fusobacterium necrophorum* subsp. *funduliforme* JCM 3724^T was a kind gift from T. Shinjo (Miyazaki University, Japan). Samples from normal horses and those with lower respiratory tract and paraoral disease were obtained from normal horses at autopsy and from horses with a range of soft-tissue infections such as paraoral abscessation, necrotizing pneumonia and pleurisy (Bailey & Love, 1991). The growth conditions, biochemical methods, DNA isolation, G+C contents of DNA and DNA–DNA hybridization methods (hybridization temperature of 56 °C) have been described previously (Love *et al.*, 1987). Enzymic activities of strains VPB 4027^T and VPB 4014 were measured by API ZYM. Cellular extracts were obtained and glutamate dehydrogenase detected as described previously (Love *et al.*, 1992) using the method of Richardson *et al.* (1986). The

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The EMBL accession number for the 16S rRNA gene sequence of strain VPB 4027^T is AJ295750.

Table 1. Levels of DNA–DNA hybridization of equine *Fusobacterium* strains, selected *Fusobacterium* type strains and reference strains and *Filifactor alocis*

Source of unlabelled DNA	Source of strain*	G + C content (mol%)	Hybridization with labelled DNA from (%):†			
			VPB 4027 ^T	VPB 4014	<i>Fusobacterium necrophorum</i> ATCC 25286 ^T	<i>Filifactor alocis</i> ATCC 35896 ^T
Equine strains						
VPB 4027 ^T	P	30	(100)	85	40	1
VPB 4013	L	30	92	92	40	9
VPB 4014	N	29	88	(100)	48	5
VPB 4017	D	ND	100	83	43	6
VPB 4018	L	30	93	92	47	4
VPB 4023	N	30	95	87	48	5
VPB 4024	L	29	76	89	36	5
VPB 4025	L	30	83	87	43	2
VPB 4026	N	ND	98	82	45	4
VPB 4076	P	30	84	77	37	4
VPB 4077	P	30	90	81	37	4
VPB 4466	N	31	91	81	36	ND
VPB 4467	N	ND	81	82	40	ND
		Means	(90)	(86)	(42)	(4)
Reference strains						
<i>Fusobacterium necrophorum</i> ATCC 25286 ^T			60	64	(100)	ND
<i>Filifactor alocis</i> ATCC 35896 ^T			5	22	ND	(100)
<i>Fusobacterium simiae</i> ATCC 33568 ^T			< 1	17		
<i>Fusobacterium necrogenes</i> ATCC 25556 ^T			2	15		
<i>Fusobacterium perfoetens</i> ATCC 2925 ^T			2	19		
<i>Fusobacterium varium</i> ATCC 8501 ^T			3	13		
<i>Fusobacterium periodonticum</i> ATCC 33963 ^T			6	15		
<i>Fusobacterium gonidiaformans</i> ATCC 25563 ^T			22	37		
<i>Fusobacterium russii</i> ATCC 2553 ^T			< 1	16		
<i>Fusobacterium nucleatum</i> ATCC 25586 ^T			< 1	7		
<i>Fusobacterium mortiferum</i> ATCC 25557 ^T			< 1	20		

ND, Not done.

* N, Normal horse pharyngeal area; P, paraoral infection; L, lower respiratory tract disease.

† Percentage reassociation values for reference DNA preparations were normalized to 100 % hybridization.

Table 2. Discriminatory characteristics for *Fusobacterium* species that convert lactate and threonine to propionate in the '*Fusobacterium necrophorum*' cluster

Taxa are listed as: 1, *Fusobacterium necrophorum* ATCC 25286^T; 2, *Fusobacterium necrophorum* subsp. *funduliforme* JCM 3724^T; 3, *Fusobacterium equinum* sp. nov.; 4, *Filifactor alocis* feline strains.

Characteristic	1	2*	3	4
Growth in bile	+	– / +	+	+
Alkaline phosphatase	+	–	–	–
Glutamate dehydrogenase mobility (cm)	3.0	3.0	2.3	1.8
Glucose fermentation	–	+ / –	–	–
Lipase	+	– / +	+	–
Haemagglutination	+	–	–	+

* Where two results are given, the first is according to Shinjo *et al.* (1991) and the second according to Bailey & Love (1993).

lipase test was carried out using the modified egg-yolk agar method of McClung & Toabe as described previously (Moore *et al.*, 1977) and haemagglutination was carried out on a white tile using 3 % washed chicken erythrocytes (Slots & Genco, 1979).

High molecular mass DNA was extracted from cells by the method of Marmur as described previously (Love *et al.*, 1987) and dialysed against 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0).

Approximately 200 ng purified DNA was used in each PCR to amplify the 16S rDNA. Amplification primers (taken from the *Escherichia coli* sequence; Woese *et al.*, 1983) were 5'-GAGTTTGATCCTGGCTCAG-3' (forward, positions 11–29) and 5'-GGGCCTTGTA-CACACCG-3' (reverse, positions 1492–1512). The reaction mixtures contained 50 mM KCl, 10 mM Tris/HCl (pH 8.4), 2.5 mM MgCl₂, 0.01 % gelatin, 0.1 μM of each primer, 0.4 mM of each nucleotide (dATP, dCTP, dGTP, dTTP) and distilled water to a

Fig. 1. Phylogenetic tree of 16S rRNA gene sequences of members of the genus *Fusobacterium* aligned with the sequence from *Fusobacterium equinum* sp. nov. Sequence accession numbers are given in parentheses. *F.*, *Fusobacterium*.

final volume of 97.5 µl. The genomic DNA was denatured by incubating the reaction mixtures for 2.5 min at 98 °C, 2.5 µl *Taq* polymerase (1 U µl⁻¹) was added and the reactions were overlaid with 80 µl light white mineral oil. PCR was done in a Perkin Elmer 480 DNA thermal cycler. The thermal profile included five cycles of annealing for 30 s at 53 °C, extension for 30 s at 72 °C and denaturation for 30 s at 94 °C, 28 cycles of annealing for 30 s at 48 °C, extension for 45 s at 72 °C and denaturation for 30 s at 94 °C, and a final extension for 2 min at 72 °C. The amount and quality of amplification product were determined by running aliquots of 10 µl on ethidium bromide-stained, 1.2% (w/v) agarose gels. A size standard was included on each gel. Products were purified using a High Pure PCR product purification kit (Roche Diagnostics) according to the supplier's instructions. Purified products were eluted with 50 µl elution buffer and aliquots of 10 µl were analysed on agarose gels as described above.

Approximately 400 ng purified PCR product was used for each sequencing reaction. Sequencing reactions were carried out with the Big Dye Terminator cycle sequencing kit (Perkin Elmer Applied Biosystems) according to the instruction manual. Sequencing primers were 5'-CTGCTGCCTCCCGTA-3' (reverse, positions 343–357), 5'-G(AT)ATTACCGCGGC-(GT)GCTG-3' (reverse, 519–536), 5'-CTACCAGG-GTATCTAAT-3' (reverse, 787–803), 5'-AGGGGT-TGCGCTCGTTG-3' (reverse, 1100–1115), 5'-CGG-

TGTGT(AG)CAAG(AG)CCC-3' (reverse, 1382–1401), 5'-CAACGAGCGCAACCCCT-3' (forward, 1100–1115) and 5'-GGG(CT)CTTG(CT)ACACAC-CG-3' (forward, 1382–1401). All sequencing primers were used at a final concentration of 3.2 pM. Sequencing reactions were analysed with an ABI Prism 377 DNA sequencer.

The 16S rDNA sequences determined for VPB 4027^T and VPB 4014 were aligned and found to be identical. The sequence was aligned with 16S rDNA sequences of 21 other species of the genus *Fusobacterium* using the CLUSTAL W program (opening gap penalty of 10 and extension penalty of 0.05) and a tree was constructed using Kimura's distance matrix algorithm and a neighbour-joining (UPGMA) method (Felsenstein, 1989) using programs accessed from BioNavigator (Entigen; <http://www.entigen.com>).

In their biochemical and other phenotypic characteristics, the horse strains resembled *Fusobacterium necrophorum* and feline strains of *Fusobacterium alocis* (Love *et al.*, 1987). *Fusobacterium alocis* has since been reassigned as *Filifactor alocis* (Jalava & Eerola, 1999). The DNA–DNA hybridization values for VPB 4027^T and VPB 4014 with *Fusobacterium necrophorum* ATCC 25286^T were 60 and 64%, respectively, and with *Filifactor alocis* ATCC 35896^T (which demonstrated a mean of 98% DNA–DNA hybridization with feline isolates of *Filifactor alocis*; Love *et al.*, 1987), they were 5 and 22%, respectively. In addition, strains VPB

4027^T and VPB 4014 respectively had DNA–DNA hybridization values of less than 22% and less than 37% with the remaining *Fusobacterium* species included in this study (Table 1). In reciprocal experiments, *Fusobacterium necrophorum* ATCC 25286^T had a mean DNA–DNA hybridization with horse isolates of 42%. In two separate experiments, *Fusobacterium necrophorum* subsp. *funduliforme* showed 49% DNA–DNA hybridization with VPB 4027^T and VPB 4027^T showed 54% DNA–DNA hybridization with *Fusobacterium necrophorum* subsp. *funduliforme*. Also, *Filifactor alocis* ATCC 35896^T had DNA–DNA hybridization values with VPB 4027^T and VPB 4014 of 1 and 5%, respectively, and a mean DNA–DNA hybridization value with equine isolates of 4%. On the basis of DNA–DNA hybridization, the equine *Fusobacterium* strains were considered to be genetically distinct from other *Fusobacterium* species included in this study. Phylogenetic analysis based on the 16S rDNA gene sequences (Fig. 1) showed that the novel strains were closely clustered with the species and biotypes of *Fusobacterium necrophorum* (Nicholson *et al.*, 1994) and *Fusobacterium gonidiaformans*, but were clearly separate from these species. These were well separated from other members of the genus *Fusobacterium*, but remained within the genus. The placement of *Filifactor alocis* and *Eubacterium sulci* as well as *Fusobacterium prausnitzii* at a distance from the members of the genus *Fusobacterium* is consistent with recent literature (Neefs *et al.*, 1993; Nicholson *et al.*, 1994; Javala & Eerola, 1999). These findings, together with the DNA–DNA hybridization values with *Fusobacterium necrophorum* ATCC 25286^T, support the inclusion of the equine strains within the genus *Fusobacterium* (Schleifer & Stackebrandt, 1983; Stackebrandt & Goebel, 1994) and their separation from any of the species described so far (Moore *et al.*, 1984; Dzink *et al.*, 1990; Shinjo *et al.*, 1991; Lawson *et al.*, 1991; Gharbia & Shah, 1992; Nicholson *et al.*, 1994; Wang *et al.*, 1996).

Description of *Fusobacterium equinum* sp. nov

Fusobacterium equinum (e.quin'um. L. adj. *equinum* of horses).

On sheep-blood agar after 2 d, colonies are circular with an entire to undulate margin, convex to umbonate, 1–2 mm in diameter and creamish in colour. Gram staining of single colonies on primary plates reveals pleomorphic Gram-negative rods (coccobacilli to rods, with rounded ends and curved rods often with irregular staining) but, on subculture, coccobacilli and short rods predominate. When subcultured to cooked-meat medium and following overnight incubation, a large amount of flocculent deposit results. In Gram staining from broth, short rods are the predominant forms. The terminal pH in glucose liquid medium ranges from pH 6.8 to 7.0. On GLC analysis, acetic, propionic and butyric acids are produced (respective means 23.1, 15.0 and 36.5 µmol ml⁻¹) and lactate and

threonine are converted to propionate. The isolates produce indole and grow in bile to 2⁺ to 4⁺ turbidity and are non-motile, fail to hydrolyse aesculin and starch and fail to liquefy gelatin or liquefy it weakly (VPB 4027^T). The isolates fail to ferment aesculin, fructose, glucose, lactose, maltose, mannose, starch and sucrose. Strains VPB 4027^T and VPB 4014 possess the constitutive enzymes acid phosphatase and phosphoamidase, butyrate esterase and caprylate esterase. Trypsin, chymotrypsin and other enzymes detected by API ZYM are not expressed constitutively in this system. They do not haemagglutinate chicken erythrocytes. Their mobility for glutamate dehydrogenase is 2.3 cm compared with 3.0 cm for *Fusobacterium necrophorum* ATCC 25286^T. They are lipase- and lecithinase-positive. On broth disc sensitivity tests, all isolates are sensitive to penicillin G, amoxycillin, chloramphenicol, doxycycline and metronidazole; all strains except VPB 4076 and VPB 4014 are sensitive to erythromycin. The G+C content of DNA is 29–31 mol% (thermal denaturation method). The mean DNA–DNA hybridization of strains VPB 4014 and VPB 4027^T with the other equine isolates is respectively 88 and 82%. With the exception of VPB 4022, the isolates have DNA–DNA hybridization with labelled DNA from either VPB 4027^T or VPB 4014 of 80%, which shows that the isolates form a tight genetic cluster. This is supported by 16S rDNA phylogenetic analysis with other members of the genus *Fusobacterium*.

The type strain is strain VPB 4027^T (= NCTC 13176^T = JCM 11174^T), which was isolated from pus obtained from a discharging sinus associated with a purulent paraoral lesion in a horse. *Fusobacterium equinum* can be distinguished phenotypically from those members of the genus *Fusobacterium* that convert threonine and lactate to propionate by a combination of characteristics included in Table 2.

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