NOTE

***Crossiella equi* sp. nov., isolated from equine placentas**J. M. Donahue,¹ N. M. Williams,¹ S. F. Sells¹ and D. P. Labeda²¹ Livestock Disease Diagnostic Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40511, USA² Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, 1815 North University Street, USDA–Agricultural Research Service, Peoria, IL 61604, USA

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Over the course of the past decade, actinomycetes have been isolated from the placentas of horses diagnosed with nocardioform placentitis. The incidence of this infection has generally been low, with typically no more than 30 animals affected in most years, but the incidence increased through 1999, with placentas from 144 mares found to be infected. Approximately half of the cases result in loss of the foal. A typical actinomycete with branching mycelium was isolated from placental lesions, and a comparison of the sequence of the 16S rDNA gene against the public databases indicated a relationship to members of the suborder *Pseudonocardineae*. Phylogenetic analysis of representative isolates revealed a close relationship to *Crossiella cryophila*, and subsequent polyphasic comparisons determined that these isolates represent a novel species of *Crossiella*, for which the name *Crossiella equi* sp. nov. is proposed, with strain LDDC 22291-98^T (= NRRL B-24104^T = DSM 44580^T) as the type strain.

Keywords: *Pseudonocardineae*, nocardioform placentitis

Nocardioform actinomycetes have been reported as a significant emergent cause of placentitis and abortion in horses in Kentucky, particularly over the past decade (Donahue & Williams, 2000; Giles *et al.*, 1993; Hong *et al.*, 1993). Nocardioform placentitis is the term used to describe this distinct type of placentitis in horses. The infection is diagnosed based on the location of the lesion on the chorionic surface of the placenta and the recovery of Gram-positive branching micro-organisms upon culture. The condition was first diagnosed in 1986 at the University of Kentucky Livestock Disease Diagnostic Center, but this type of placentitis has not been confirmed elsewhere in the USA or in other countries.

The 16S rDNA of strains of the causative micro-organism most commonly isolated was sequenced and subsequently found to be phylogenetically most closely related to *Crossiella cryophila* NRRL B-16238^T, a recently described species in a new genus within the *Pseudonocardineae* (Labeda, 2001). A polyphasic study was undertaken to characterize these strains and to determine their taxonomic position in the genus

Crossiella and, based on the findings reported, we propose a novel species of the genus *Crossiella*, *Crossiella equi* sp. nov.

Strains were originally isolated from placental tissues on tryptic soy agar (Difco) plus 5% blood, but all grow well on media typically used to culture actinomycetes and have been accessioned into the ARS Culture Collection (National Center for Agricultural Utilization Research, Peoria, IL) as NRRL B-24102 (= LDDC 5832-88), NRRL B-24103 (= LDDC 6743-88), NRRL B-24104^T (= LDDC 22291-98^T = DSM 44580^T), NRRL B-24105 (= LDDC 68043-98), NRRL B-24106 (= LDDC 69281-98) and NRRL B-24107 (= LDDC 6831-99).

Gross morphological observations were made using cultures grown for 14 days at 28 °C on the standard media suggested by the International *Streptomyces* Project (Shirling & Gottlieb, 1966) and on Czapek's sucrose agar (Pridham & Lyons, 1980). Micromorphology and sporulation were observed by light microscopy and scanning electron microscopy (SEM). Samples for SEM observation were 14-day cultures on agar media that were fixed and observed as described previously (Labeda, 2001). Chemotaxonomic analysis of strains for menaquinones, fatty acids, cell-wall diamino acid and whole-cell sugars was performed on formalin-killed biomass using methods described pre-

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The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain NRRL B-24104^T is AF245017.

Fig. 1. Radial phylogenetic tree for the families of the suborder *Pseudonocardineae* calculated from almost-complete 16S rDNA sequences using Kimura's evolutionary distance methods (Kimura, 1980) and the neighbour-joining method of Saitou & Nei (1987), illustrating the taxonomic position of *Crossiella equi* sp. nov. NRRL B-24104^T relative to *C. cryophila* NRRL B-16238^T and the other taxa within the suborder. The taxa and sequences included in the genus groups are: *Actinokineospora diospyrosa* NRRL B-24047^T, AF114797; *Actinokineospora globicatena* NRRL B-24048^T, AF114798; *Actinokineospora inagensis* NRRL B-24050^T, AF114799; *Actinokineospora riparia* NRRL B-16432^T, AF114802; *Actinosynnema mirum* DSM 43827^T, X84447; *Actinosynnema pretiosum* subsp. *pretiosum* NRRL B-16060^T, AF114800; *Amycolatopsis sacchari* DSM 44468^T, AF223354; *Amycolatopsis albidoflavus* IMSNU 22139^T, AJ252832; *Amycolatopsis azurea* NRRL 11412^T, X53199; *Amycolatopsis coloradensis* DSM 44225^T (= NRRL 3218^T), AF051340; '*Amycolatopsis keratinophila*' DSM 44409, AJ278496; *Amycolatopsis mediterranei* ATCC 13685^T, X76957; *Amycolatopsis methanolica* NCIB 11946^T, X54274; *Amycolatopsis orientalis* subsp. *orientalis* DSM 40040^T, X76958; *Amycolatopsis rubida* JCM 10871^T, AF222022; *Amycolatopsis alba* DSM 44262^T, AF051340; *Amycolatopsis thermoflava* IFO 14333^T, AF052390; *Lechevalieria aerocolonigenes* NRRL B-3298^T, AF114804; *Lechevalieria flava* NRRL B-16131^T, AF114808; *Lentzea albida* IFO 16102^T, AB006176; *Lentzea albidocapillata* DSM 44073^T, X84321; *Lentzea californiensis* NRRL B-16137^T, AF174435; *Lentzea violacea* IMSNU 50388^T, AJ2242633; *Lentzea waywayandensis* NRRL B-16159^T, AF114813; *Pseudonocardia alni* VKM Ac-901^T, X76954; *Pseudonocardia autotrophica* DSM 43210, X54288; *Pseudonocardia compacta* DSM 43592^T, X76959; *Pseudonocardia halophobica* DSM 43089^T, Z14111; *Pseudonocardia hydrocarbonoxydans* DSM 43281^T, X76955; *Pseudonocardia nitrificans* IFAM 379^T, X55609; *Pseudonocardia petroleophila* ATCC 15777^T (= DSM 43193^T), X80596; *Pseudonocardia saturnea* DSM 43195^T, X76956; *Pseudonocardia thermophila* ATCC 19285^T, X53195; *Saccharomonospora azurea* INMI 19125, X76960; *Saccharomonospora viridis* Goodfellow SB-33, X54286; *Saccharopolyspora erythraea* NRRL 2338^T, X53198; *Saccharopolyspora gregorii* NCIB 12823^T, X76962; *Saccharopolyspora hirsuta* ATCC 27875^T, X53196; *Saccharopolyspora hordei* ATCC 49856^T, X53197; *Saccharopolyspora rectivirgula* ATCC 33515^T, X53194; *Saccharothrix australiensis* NRRL 11239^T, AF114803; *Saccharothrix coeruleofusca* NRRL B-16115^T, AF114805; *Saccharothrix espanaensis* NRRL 15764^T, AF114807; *Saccharothrix longispora* NRRL B-16116^T, AF114809; *Saccharothrix mutabilis* subsp. *capreolus* DSM 40225^T, X76965; *Saccharothrix mutabilis* subsp. *mutabilis* DSM 43853^T, X76966; *Saccharothrix syringae* NRRL B-16468^T, AF114812; *Saccharothrix texasensis* NRRL B-16134^T, AF114814; *Thermocrispum agreste* DSM 44070^T, X79183; *Thermocrispum municipale* DSM 44069^T, X79184. Bar, 0.1 nucleotide substitutions per site.

viously (Grund & Kroppenstedt, 1989; Meyerton *et al.*, 1988; Staneck & Roberts, 1974). Physiological tests were evaluated by using the media of Gordon *et al.* (1974). Allantoin hydrolysis was evaluated in the basal medium suggested by Gordon *et al.* (1974) for aesculin hydrolysis. Phosphatase activity was evaluated by using the method of Kurup & Schmitt

(1973). The temperature range for growth was determined on slants of ATCC medium 172 agar (Cote *et al.*, 1984). Genomic DNA from each isolate was purified using the High Pure PCR template preparation kit (Roche Diagnostics) following the manufacturer's instructions. The 16S rDNA was amplified, purified and then sequenced using the MicroSeq 16S

Fig. 2. Comparison of the micromorphological properties of *C. equi* sp. nov. NRRL B-24104^T (a, c) and *C. cryophila* NRRL B-16238^T (b, d). Note the swollen mycelial tips in (a) and (b) and the pseudosporangia on the substrate mycelium in (c) and (d). Bars, 1 µm.

rRNA gene kit (Applied Biosystems). Amplified 16S rDNA products were purified prior to the sequencing reactions using a QIAquick PCR purification kit (Qiagen). The sequencing reactions for each isolate were purified and sequenced at the University of Kentucky Molecular Structure Analysis Facility. Data were assembled and edited using Sequencher 4.0 (Gene Codes Corporation). A phylogenetic tree was constructed within the ARB software environment for sequence data developed by W. Ludwig and O. Strunk (Lehrstuhl für Mikrobiologie, University of Munich, Germany) using evolutionary distances by the method of Kimura (1980) and linkages by the neighbour-joining method of Saitou & Nei (1987). Genomic DNA was isolated and DNA relatedness between strain NRRL B-24104^T and *Crossiella cryophila* NRRL B-16238^T was determined as described previously (Labeda, 1998).

BLAST searches of the 16S rDNA gene sequences of the equine isolates had previously indicated a strong relationship to members of the family *Actinosynnemataceae*, and phylogenetic analysis of these sequences and those of the suborder *Pseudonocardineae* demonstrated that these strains are phylogenetically very closely related to *C. cryophila* NRRL B-16238^T, as shown in the radial phylogenetic tree in Fig. 1. There is 98.1% 16S rDNA sequence similarity between the

equine isolates and *C. cryophila*, and DNA relatedness between NRRL B-24104^T and *C. cryophila* NRRL B-16238^T was determined to be 28%. This indicates that the equine isolates are not members of *C. cryophila*, but that they belong to the genus *Crossiella*.

Observation of the gross morphological characteristics of the equine strains further supported their assignment to the genus *Crossiella* because of the presence of bulbous swollen regions on the hyphae on the substrate mycelium of the equine isolates (Fig. 2a) as well as *C. cryophila* NRRL B-16238^T (Fig. 2b). Moreover, pseudosporangia characteristic of the genus *Crossiella* (Fig. 2d) were also observed on the substrate mycelium of the equine isolates (Fig. 2c).

The cell chemistry of the equine isolates is also characteristic of the genus *Crossiella*, with *meso*-diaminopimelic acid as the cell-wall diamino acid, a whole-cell sugar pattern consisting of galactose, mannose, rhamnose and ribose and a phospholipid pattern consisting of phosphatidyl ethanolamine, diphosphatidyl glycerol, phosphatidyl inositol, phosphatidyl inositol mannosides and phosphatidyl methylethanolamine. The predominant menaquinone was observed to be MK-9(H₄), as found in *C. cryophila* NRRL B-16238^T. Fatty acid profiles of the equine isolates exhibit fatty acids similar to those of *C. cryophila* NRRL B-

Table 1. Fatty acid profiles of *Crossiella* species

Values are percentages of total fatty acids.

Fatty acid	<i>C. equi</i> (6 strains)	<i>C. cryophila</i> NRRL B-16238 ^T
iso-14:0	1.02	1.91
iso-15:0	57.51	44.28
anteiso-15:0	3.01	2.29
B-15:1	3.34	3.06
iso-H-16:1	1.98	3.16
iso-16:0	7.25	9.77
16:1 ω 9c	0.80	3.11
16:0	0.66	1.76
10-methyl-iso-16:0	0.00	1.09
9?-methyl-16:0	4.20	11.58
iso-17:0	4.54	11.36
anteiso-17:0	1.05	1.86
17:1 ω 9c	0.82	1.86
17:0	0.50	1.21
iso-17:0 3-hydroxy	4.68	0.00

16238^T (Table 1), but the differences observed in percentages of components reflect that these strains represent another species in this genus.

The equine isolates can be clearly differentiated from *C. cryophila* NRRL B-16238^T on the basis of physiological properties such as the ability to grow at 37 °C and production of acid from a number of sugars, as shown in Table 2. Detection of the production of acid from carbohydrates and assimilation of organic acids was made difficult by the unusual ability of the isolates to absorb the pH indicator (bromothymol blue) from the agar medium into their substrate mycelium.

The data support the conclusion that the equine isolates are members of the genus *Crossiella* and clearly represent a novel species, for which the name *Crossiella equi* sp. nov. is proposed.

Description of *Crossiella equi* sp. nov.

Crossiella equi (e'qui. L. gen. n. *equi* of the horse, referring to the source of isolation of this micro-organism, equine placentas).

Pale orange to light-brown substrate mycelium is produced on most media. Copious white aerial mycelium is produced. Chemotaxonomic characteristics correspond to those typical for the genus *Crossiella*. Casein, aesculin, starch and tyrosine are hydrolysed or decomposed. Adenine, allantoin, xanthine and urea are not hydrolysed or decomposed. Gelatin, hypoxanthine and hippurate are hydrolysed variably. Nitrate is reduced to nitrite. Phosphatase is produced. Acetate, benzoate, citrate, propionate and succinate are viably assimilated; lactate, malate, mucate, oxalate and DL-tartrate are not assimilated. Acid is produced from cellobiose, dextrin, D-fructose, D-galactose, D-glucose,

Table 2. Differential physiological characteristics of *Crossiella* species

v, Variable reaction.

Characteristic	<i>C. equi</i>		<i>C. cryophila</i> NRRL B-16238 ^T
	6 strains	NRRL B-24104 ^T	
Hydrolysis of:			
Casein	+	+	—
Gelatin	+	(4)	—
Hypoxanthine	—	(4)	+
Urea	—	(5)	—
Hippurate	+	(5)	—
Assimilation of:			
Acetate	v	(5)	v
Benzoate	v	(4)	v
Citrate	v	(3)	v
Lactate	—	(4)	v
Malate	—	—	+
Oxalate	—	—	+
Propionate	v	(3)	v
Succinate	v	(4)	v
Acid from:			
Arabinose	+	(3)	—
Cellobiose	+	+	+
Dextrin	+	+	—
Mannitol	—	(4)	v
Raffinose	+	+	+
Rhamnose	+	(5)	+
Salicin	+	(5)	+
Acid from:			
Sorbitol	—	(5)	—
Sucrose	—	(5)	—
Xylose	+	(5)	—
Growth at 37 °C	+	+	+

glycerol, *myo*-inositol, maltose, D-mannose, melibiose and raffinose; no acid is produced from adonitol, dulcitol, *meso*-erythritol, mannitol, methyl α -D-glucoside, methyl α -D-xyloside, D-sorbitol or sucrose; acid production is variable from arabinose, cellobiose, lactose, rhamnose, salicin, trehalose and xylose. Growth occurs in the presence of 5% (w/v) NaCl. The temperature range for growth is 10–42 °C. The G + C content of the DNA is 71.4 mol% (thermal denaturation midpoint method). Isolated from equine placenta in Lexington, KY, USA. Implicated in equine placentitis and spontaneous abortions in mares. The type strain is strain LDDC 22291-98^T (= NRRL B-24104^T = DSM 44580^T).

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