Orbus hercynius gen. nov., sp. nov., isolated from faeces of wild boar, is most closely related to members of the orders 'Enterobacteriales' and Pasteurellales

Maria Volkmann,¹ Evelyn Skiebe,¹ Tobias Kerrinnes,¹ Franziska Faber,¹ Daniela Lepka,¹ Yvonne Pfeifer,¹ Gudrun Holland,² Norbert Bannert² and Gottfried Wilharm¹

Correspondence
Gottfried Wilharm
wilharmg@rki.de

¹Robert Koch-Institute, Wernigerode Branch, Burgstr. 37, D-38855 Wernigerode, Germany

²Robert Koch-Institute, ZBS 4, Nordufer 20, D-13353 Berlin, Germany

A novel gammaproteobacterium, strain CN3^T, was isolated from the faeces of wild boar. Strain CN3^T was facultatively anaerobic and appeared coccoid or rod-shaped. The partial 16S rRNA gene sequence determined for strain CN3^T suggested a distant relationship with members of the orders 'Enterobacteriales' and Pasteurellales. The gene sequence showed highest similarity (90.3%) with *Obesumbacterium proteus* DSM 2777^T, a member of the family Enterobacteriaceae. The closest relatives outside the order 'Enterobacteriales' according to 16S rRNA gene sequence analysis were members of the order Pasteurellales with 88.7% similarity (*Mannheimia haemolytica* NCTC 9380^T and *Actinobacillus lignieresii* NCTC 4189^T). In contrast to most members of the order 'Enterobacteriales', strain CN3^T was oxidase-positive. The pattern of fatty acids, in particular the high relative abundance of C_{18:1ω7c} (38.5%), was clearly distinct from the conserved pattern found for members of the order Pasteurellales. *EcoRI* ribotyping of strain CN3^T yielded no significant similarity to existing database entries. The major ubiquinone of strain CN3^T was Q-8. The DNA G + C content was 36.4 mol%. Strain CN3^T hosted a phage and secreted considerable amounts of three proteins into the culture supernatant. A spontaneous mutant of strain CN3^T was isolated which formed long filaments. Microscopic studies revealed the presence of a capsule that the mutant strain was unable to partition after cell division. Strain CN3^T thus represents a novel species within a new genus, for which the name *Orbus hercynius* gen. nov., sp. nov. is proposed. The type strain of the type species is CN3^T (=DSM 22228^T=CCUG 57622^T). Classification of the novel species to the family and order level will require further investigations.

In search of *Yersinia enterocolitica*, which is frequently isolated from swine, samples of wild boar faeces were collected from the Halberstadt Zoo, Germany (51° 53' 45" N, 11° 2' 48" E). Fresh faecal samples collected from the ground were suspended in sterile water and suspensions were plated on cefsulodin–irgasan–novobiocin (CIN) agar plates (Schiemann, 1979). After incubation at 27 °C for 40 h, *Yersinia*-like colonies with a 'bull's-eye'

appearance were subjected to PCR using 16S rRNA gene primers specific for European bio-serotypes of *Y. enterocolitica* (Trebesius *et al.*, 1998). For images of the colonies on CIN plates, see Supplementary Fig. S1 in IJSEM Online. Since no PCR-product was obtained from the *Yersinia*-like colonies derived from one of the faecal samples, an approximately 1500 bp fragment of the 16S rRNA gene of the representative isolate, strain CN3^T, was amplified using primers designed for members of the family Enterobacteriaceae and relatives (primers fD2 and rP1 according to Weisburg *et al.*, 1991) and was subsequently sequenced. To confirm the DNA sequencing results, the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Dr Cathrin Spröer, DSMZ) was commissioned to perform a 16S rRNA gene sequence analysis on strain CN3^T. The partial 16S

Abbreviations: SEM, scanning electron microscopy; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CN3^T is FJ612598.

Supplementary figures showing the morphology, growth and motility of strain CN3^T and the SDS-PAGE analysis are available with the online version of this paper.

rRNA gene sequence determined, encompassing 1522 bp of strain CN3^T, showed the highest sequence similarity, 90.3 %, with *Obesumbacterium proteus* DSM 2777^T, a member of the family *Enterobacteriaceae*. Many other members of the family *Enterobacteriaceae*, as well as representatives of the genera *Salmonella*, *Shigella*, *Klebsiella*, *Yersinia*, *Enterobacter* and others, showed gene sequence similarities in the range 89 to 90 %. The closest relatives outside the order 'Enterobacteriales', according to 16S rRNA gene sequence analysis, were members of the order *Pasteurellales* with 88.7 % similarity (*Mannheimia haemolytica* NCTC 9380^T and *Actinobacillus lignieresii* NCTC 4189^T). To illustrate the phylogenetic relationships of strain CN3^T, a neighbour-joining tree is presented in Fig. 1.

From the 16S rRNA gene sequence determined for CN3^T, primers were constructed for the amplification of a 16S rRNA gene fragment. The sequence of the selected forward primer 5'-TATGGAGTGTGGGGGCATGAC-3' (CN3^T-ident-for) was unique among all nucleotide database entries. Only a few database entries were found with identical 3' ends so that high stringency of the PCR conditions allowed the specificity of the PCR to be controlled. Reverse primer 5'-GTCCGCTCCAGTTCGCACC-3' (CN3^T-ident-rev) was less specific, but the sequence was found in only a very few other bacteria. A PCR protocol using crude bacterial lysates and with total isolated DNA from faeces (isolated with QIAamp DNA Stool Mini kit from Qiagen according to the manufacturer's instructions) was established which yielded a specific product of 457 bp but never yielded unspecific products. PCR conditions were 30 cycles

with 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C after an initial denaturation step at 94 °C for 2 min. By applying this PCR protocol, more than 20 faecal samples from wild boar were screened for the presence of relatives of strain CN3^T but the strain was not detected in any other sample. Further parallel trials to isolate strains related to CN3^T from the same samples by applying culture techniques also failed.

Phenotypic and cultural characteristics

The cell morphology of strain CN3^T was examined by scanning and transmission electron microscopy (SEM, TEM; Fig. 2). Strain CN3^T appeared coccoid to rod-shaped with typical dimensions of 1–1.5 × 0.8 µm (Fig. 2a). TEM further revealed that strain CN3^T hosted a phage that was morphologically related to the Myoviridae (Fig. 2b). Since no flagella and no swimming motility could be observed, the novel strain was tested on semi-solid media for surface-associated forms of motility (see Supplementary Fig. S2 in IJSEM Online). Strain CN3^T was inoculated on 0.3 % agar plates containing 2 × YT broth (16 g l⁻¹ tryptone, 10 g l⁻¹ yeast extract, 5 g l⁻¹ NaCl) by puncturing the agar and was then incubated for 7 days at 27 °C. Drying was prevented by wrapping with Parafilm. Strain CN3^T was found to move slowly on the agar surface with a variable morphological appearance. Movement of strain CN3^T could also be observed at the boundary between the bottom of the agar layer and the polystyrene Petri-dish (referred to here as the 'interphase'; see Supplementary Fig. S2). To visualize the biofilm at the interphase, the agar

Fig. 1. Neighbour-joining phylogenetic reconstruction from almost complete 16S rRNA gene sequences using the ARB package (Pruesse *et al.*, 2007) and the correction of Jukes & Cantor (1969). The root of the tree was determined by including the 16S rRNA gene sequence of *Vibrio furnissii* in the analysis. Bar, 1 nt substitution per 100 nt.

Fig. 2. (a) SEM of cells of strain CN3^T after cultivation on 2×YT agar for 40 h at 27 °C. (b) TEM (negative staining) of a phage of strain CN3^T. Bars, 1 µm (a), 200 nm (b).

layer was removed and bacteria attached to the polystyrene surface were stained with Coomassie blue (see Supplementary Fig. S2). One surface-associated form of motility, termed twitching motility, has been associated with polarly localized type IV pili in several bacteria (Mattick, 2002). However, TEM and SEM examinations did not point to such a mechanism for strain CN3^T, leaving open the mechanism behind this form of motility.

When inspecting motility by light microscopy, a spontaneous filamentous mutant was identified on semi-solid agar (0.3 %) and characterized by light and electron microscopy (see Supplementary Fig. S3). Filaments of at least 50 µm in length were observed which appeared to be coated by a capsule-like sheath. Bacteria had escaped from some filament segments, leaving behind the empty sheath (see Supplementary Fig. S3), which collapsed during sample preparation for SEM resulting in a beading phenotype (see Supplementary Fig. S2). Interestingly, some of the beads had a spore-like appearance with a diameter exceeding that of the filament. It remains to be determined whether these beads are of biological relevance or whether they represent artefacts.

Strain CN3^T grew well in 2 × YT broth at 27 °C; growth was suboptimal in Luria–Bertani (Alpha Biosciences) and brain heart-infusion (Becton Dickinson) media. Besides growth on CIN agar plates (Supplementary Fig. S1), strain CN3^T grew on bile–chrysoidine–glycerol agar (GCG agar, Heipha Diagnostika; Ziesché *et al.*, 1985) forming small grey colonies after incubation for 48 h at 27 °C. The optimum temperature for growth of strain CN3^T was tested in 2 × YT broth, both under aerobic and anaerobic conditions (paraffin overlay) in the range 16 to 40 °C (see Supplementary Fig. S4). After incubation for 18 h, a broad temperature optimum was found between 20 and 30 °C with a sharp drop in growth above 36 °C and below 20 °C, both under aerobic and anaerobic conditions. However, significant growth was also observed at 4 °C (incubation in 2 × YT broth for 7 days without shaking).

The pH-dependent growth range was tested in buffered 2 × YT broth at 22 °C at between pH 4 and 10. Significant growth of strain CN3^T was observed between pH 6 and pH 8 with an optimum around pH 7.5.

In the API 20NE test system, strain CN3^T showed nitrate reduction, glucose fermentation, urease activity and

aesculin hydrolysis after incubation at 30 °C for 48 h. No arginine dihydrolase activity, indole production, gelatin hydrolysis or β-galactosidase activity was observed. Assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, gluconate, capric acid, adipic acid, malic acid, citrate or phenylacetic acid were all negative for strain CN3^T as shown with the API 20 NE system.

Chemotaxonomic properties

Analysis of respiratory quinones was carried out by Dr Brian Tindall of the DSMZ Identification Service, Germany. The major ubiquinone of strain CN3^T was Q-8. Since Q-8 is also the major ubiquinone of some members of the family *Pasteurellaceae*, as well as of some members of the family *Enterobacteriaceae* and other gammaproteobacteria, this result did not allow further classification of strain CN3^T.

Ribotyping was performed with the Qualicon RiboPrinter system by Dr Peter Schneider of the DSMZ Identification Service. The *EcoRI* riboPrint pattern showed no significant similarity (>0.85) to the entries in the DuPont identification library or to entries in the internal DSMZ database.

The DNA base composition (G + C content) of strain CN3^T was determined by Dr Peter Schumann of the DSMZ Identification Service, following the procedure described by Mesbah *et al.* (1989). The G + C content of strain CN3^T was 36.4 mol% (two independent determinations). For comparison, the DNA G + C content of members of the family *Enterobacteriaceae* is typically in the range 45 to 55 %, with the exception of *Proteus mirabilis* (38.9 %) (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>). Members of the family *Pasteurellaceae* typically have DNA G + C contents of around 40 % (*Actinobacillus* 41–44 %, *Haemophilus* 37–40 %, *Mannheimia* 43 %) (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>).

No plasmids could be identified using standard plasmid purification protocols.

Fatty acids were extracted and analysed by the Identification Service of the DSMZ according to the standard protocol of the Microbial Identification System (MIDI Microbial ID Inc.) by using the TSB40 method. The major fatty acids determined for strain CN3^T were C_{18:1ω7c}

(38.45 %) and C_{16:0} (33.73 %) (Table 1). It is important to note that fatty acid profiles are highly conserved among members of the family *Pasteurellaceae* with C_{18:1ω7c} not being a regular constituent (Mutters *et al.*, 1993; Christensen *et al.*, 2007).

Strain CN3^T does not interact with HeLa cells

HeLa cells were cultured in EMEM medium (Cell Concepts) supplemented with 1 % non-essential amino acids, 2 mM glutamine and 5 % heat-inactivated fetal calf serum (FCS). For infection experiments, eukaryotic cells were cultivated in SonicSeal slide wells (Nunc). After overnight culture of strain CN3^T in 2 × YT or on 2 × YT agar plates (27 °C), HeLa cells were infected with strain CN3^T (m.o.i. 50) and infected cultures were further incubated for 3 h at 37 °C in the presence of 5 % CO₂. The wells were then washed five times with PBS (8 g l⁻¹ NaCl, 1.15 g l⁻¹ Na₂HPO₄, 0.2 g l⁻¹ KCl, 0.2 g l⁻¹ KH₂PO₄) and cells were fixed in methanol for 5 min, stained with Accustain modified Giemsa stain (Sigma) and examined under a light microscope. Neither significant adherence of strain CN3^T to HeLa cells nor any cytopathic effects could be observed. Given the poor growth of strain CN3^T at 37 °C and the lack of evidence for interaction with HeLa cells, we do not expect the novel strain to be a potential pathogen of endotherms. Therefore, isolation of this novel strain from wild boar faeces was probably due to accidental intestinal passage after uptake from soil or feed or due to the contamination of the faecal samples that were collected from the ground. This observation was in agreement with our unsuccessful efforts to isolate organisms related to strain CN3^T from any other faecal sample.

Secreted proteins

We also analysed whether strain CN3^T secreted proteins into the culture supernatant. Single colonies were

suspended in 3 ml 2 × YT broth and cultured for 24 h at 27 °C under aerobic and anaerobic conditions, respectively, as described above. Fractions of 2 ml were then centrifuged for 10 min at 10 000 g to pellet the bacteria. A 1.7 ml sample of cleared supernatant was then transferred to a new tube for the precipitation of proteins with 200 µl trichloroacetic acid. After 1 h incubation on ice, samples were centrifuged for 30 min at 4 °C (14 000 g) and the supernatants were discarded after centrifugation. The pellets were washed twice with ice-cold acetone and subsequently air-dried. Pellets were dissolved in 30 µl SDS-PAGE loading buffer (Trček *et al.*, 2002) and 10 µl samples were subjected to SDS-PAGE analysis (Laemmli, 1970). Strain CN3^T secreted three proteins with approximate molecular masses of 55, 37 and 23 kDa at considerable amounts (each at approx. 0.5–2 mg l⁻¹) irrespective of the presence of oxygen (see Supplementary Fig. S5 in IJSEM Online).

Resistance to antibiotics

In a standard microbroth dilution assay conducted according to established protocols (NCCLS, 1997, 1999), resistance to the following commonly used antibiotics belonging to different antibiotic classes was determined: ampicillin, mezlocillin, mezlocillin–sulbactam, cefotiam, cefotaxime, ceftazidime, cefoxitin, gentamicin, kanamycin, amikacin, streptomycin, nalidixic acid, chloramphenicol, oxytetracycline, ciprofloxacin, sulfameracin, sulfameracin–trimethoprim. Strain CN3^T was sensitive to all antibiotics with the exception of ampicillin and cefoxitin, for which minimal inhibitory concentrations of 8 and 2–16 mg ml⁻¹, respectively, were determined, reflecting intermediate resistance.

Classification

Strain CN3^T is considered to represent a novel species and genus within the class *Gammaproteobacteria*. This proposal is substantiated by its isolated phylogenetic position according to 16S rRNA gene sequence analysis, the presence of the oxidase reaction in contrast to most members of the order 'Enterobacteriales', the high relative abundance of fatty acid C_{18:1ω7c} in contrast to the order *Pasteurellales*, and the distinct ribotype pattern. The name *Orbus hercynius* gen. nov., sp. nov. is proposed. Determination of the exact taxonomic standing within the class *Gammaproteobacteria* will require further studies and probably the description of a novel family and order.

Description of *Orbus* gen. nov.

Orbus (Or'bus. L. masc. n. *orbus* orphan).

Mesophilic, psychrotolerant, chemoheterotrophic bacteria. Metabolism is aerobic and facultatively anaerobic. Major fatty acids are monounsaturated, even-numbered, straight-chain C₁₈ (C_{18:1ω7c}) and saturated, even-numbered,

Table 1. Fatty acid composition of strain CN3^T

Fatty acid	Proportion (%)
C _{10:0}	0.08
C _{12:0}	0.16
C _{14:0}	6.88
Unknown 14.502	0.10
C _{16:1ω5c}	0.17
C _{16:0}	33.73
C _{18:1ω7c}	38.45
C _{18:0}	0.35
Summed features*	
2	9.37
3	10.70

*Summed feature 2 comprises iso-C_{16:1} I, C_{14:0} 3-OH and/or C_{12:0} aldehyde; summed feature 3 comprises C_{16:1ω7c} and/or iso-C_{15:0} 2-OH.

straight-chain C₁₆ (C_{16:0}) fatty acids. Cells are coccoid or rod-shaped, Gram-negative, oxidase-positive and catalase-positive. The type species is *Orbus hercynius*.

Description of *Orbus hercynius* sp. nov.

Orbus hercynius (her.cy'ni.us. L. masc. adj. *hercynius* pertaining to Hercynia, N.L. name of the Harz Mountains, Germany).

Displays the following properties in addition to those described above for the genus. Cells are coccoid or short rods, 0.8 µm wide, 1–1.5 µm long and coated with a capsule-like sheath. Cells carry no flagella. Flagella-independent motility alongside wet surfaces can be observed. Pigments are not produced. Colonies show a 'bull's-eye' appearance on CIN agar plates and grow up to 1–2 mm in diameter on CIN agar. Growth at 4–37 °C with optimal growth under aerobic conditions at 20–30 °C. The API 20NE test system shows positive reactions for nitrate reduction, glucose fermentation, urease activity and aesculin hydrolysis. Arginine dihydrolase activity, indole production, gelatin hydrolysis and β-galactosidase activity are not observed. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, gluconate, capric acid, adipic acid, malic acid, citrate or phenylacetic acid is shown with the API 20 NE system. Major fatty acids are C_{18:1ω7c}, C_{16:0}, summed feature 2 (iso-C_{16:1} I, C_{14:0} 3-OH and/or C_{12:0} aldehyde), summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH) and C_{14:0}. The major ubiquinone is Q-8.

The type strain, CN3^T (=DSM 22228^T=CCUG 57622^T), was isolated from faeces of wild boar collected at the Halberstadt Zoo, Germany. The G + C content of the DNA of the type strain is 36.4 mol%.

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