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Orbus hercynius gen. nov., sp. nov., isolated from faeces of wild boar, is most related to *Enterobacteriales* and *Pasteurellales*

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Running title:

Orbus hercynius gen. nov., sp. nov.

Subject category: New Taxa

Subsection: *Proteobacteria*

The GenBank accession number for the 16S rRNA gene sequence of strain CN3^T (=DSM 22228^T=CCUG 57622^T) is FJ612598.

SUMMARY

A novel gammaproteobacterium, strain CN3^T, was isolated from faeces of wild boar. It is facultative anaerobic and appears coccoid or rod shaped. The determined partial 16S rRNA gene sequence of strain CN3^T suggests a distant relationship to *Enterobacteriales* and *Pasteurellales*. The sequence shows highest similarity of 90.3% with *Obesumbacterium proteus* DSM 2777^T, a member of the *Enterobacteriaceae*. The closest relatives outside the *Enterobacteriales* according to 16S rRNA gene sequence analysis are members of the *Pasteurellales* with 88.7% similarity (*Mannheimia haemolytica* NCTC 9380^T and *Actinobacillus lignieresii* NCTC 4189^T). In contrast to most members of the *Enterobacteriales*, strain CN3^T is oxidase-positive. The pattern of fatty acids, in particular the high relative abundance of $C_{18:1}\omega7c$ (38.5%), is clearly distinct from the conserved pattern of *Pasteurellales*. *Eco*RI ribotyping of strain CN3^T yielded no significant similarity to database entries. Major ubiquinone of strain CN3^T is Q-8. The DNA G+C content is 36.4 mol%. CN3^T hosts a phage and secretes considerable amounts of three proteins into the culture supernatant. A spontaneous mutant of strain CN3^T was isolated forming long filaments. Microscopic studies revealed the presence of a capsule which the mutant strain is unable to partition after cell division. CN3^T (=DSM 22228^T=CCUG 57622^T) is considered as the type strain of a novel species within a new genus, for which the name *Orbus hercynius* gen. nov., sp. nov. is proposed. Its classification to family and order requires further investigation.

In search of *Yersinia enterocolitica*, which is frequently isolated from swine, wild boar faeces was collected at the zoo of Halberstadt, Germany (zip code D-38820, Spiegelsberge 4, Harz district, Saxony-Anhalt, coordinates: 51°53′45″N 11°2′48″E). Fresh faeces 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 hours, *Yersinia*-like colonies with 'bulls-eye' appearance were subjected to PCR using 16S rRNA gene primers specific for european bio-/serotypes of *Y. enterocolitica* (Trebesius *et al.*, 1998). Since we yielded no PCR-product from *Yersinia*-like colonies derived from one of the faeces samples, an approximately 1500 bp fragment of the 16S rRNA gene of the representative isolate, strain CN3^T (Supplementary Fig. S1), was amplified using primers designed for *Enterobacteriaceae* and relatives (primers fD2 and rP1 according to Weisburg *et al.*, 1991) and was subsequently sequenced. To confirm our DNA sequencing results, the Identification service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany; Dr. Cathrin Spröer) was commissioned also to perform a 16S rRNA gene sequence analysis on strain CN3^T. The determined partial 16S rRNA gene sequence encompassing 1522 bp of strain CN3^T (deposited at GenBank under accession no. FJ612598) shows highest similarity of 90.3% with *Obesumbacterium proteus* DSM 2777^T, being a member of the *Enterobacteriaceae*. Many other *Enterobacteriaceae*, representatives of the genera *Salmonella*, *Shigella*, *Klebsiella*, *Yersinia*, *Enterobacter* and others, show similarities in the range between 89 and 90%. The closest relatives outside the *Enterobacteriales* according to 16S rRNA gene sequence analysis are members of the *Pasteurellales* with 88.7% similarity (*Mannheimia haemolytica* NCTC 9380^T and *Actinobacillus lignieresii* NCTC 4189^T). To illustrate the phylogenetic relationship, a neighbour-joining tree is given in Fig. 1.

From the determined 16S rRNA gene sequence of CN3^T we deduced primers for 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 few database entries were found with identical 3' ends so that high stringency of PCR conditions should allow to control specificity of the PCR reaction. Reverse primer 5'-GTCCGCTCCAGTTCGCACC-3' (CN3^T-ident-rev) was less specific but the sequence was found in only very few other bacteria. We established a PCR protocol working with 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) which yielded a specific product of 457 base pairs 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 minutes. Applying this PCR protocol, we screened more than 20 faeces samples from wild boar for the presence of relatives of strain CN3^T but failed to detect it in any other sample. In accordance, paralleled trials to isolate strains related to CN3^T from the same samples applying culture techniques also failed.

Phenotypic and cultural characteristics

Cell morphology of CN3^T was examined by scanning and transmission electron microscopy as depicted in Fig. 2. Strain CN3^T appears coccoid to rod-shaped with typical dimensions of 1-1.5 x 0.8 µm (Fig. 2A). Transmission electron microscopy further revealed that strain CN3^T hosts a phage morphologically related to the Myoviridae (Fig. 2B). Since no flagella and no swimming motility could be observed, we tested on semi-solid media for surface-associated forms of motility (Supplementary Fig. S2). CN3^T was inoculated on 0.3% agar plates containing 2xYT broth by puncturing the agar and incubated for 7 days at 27°C, preventing drying by parafilm wrapping. CN3^T was found to slowly move on the agar surface with

variable morphological appearance. Also, at the boundary between the bottom of the agar layer and the polystyrene Petri-dish (here to be termed "interphase"), movement of CN3^T could be observed (Supplementary Fig. S2). To visualize the biofilm at the interphase, the agar layer was removed and bacteria attaching to the polystyrene surface were stained with Coomassie blue (Supplementary Fig. S2). One surface-associated form of motility, termed twitching motility, has been implicated with polarly localized type IV pili in several bacteria (Mattick, 2002). However, transmission and scanning electron microscopy examinations did not point to such a coherency, 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 characterised by light and electron microscopy (Supplementary Fig. S3). We observed filaments with at least 50 μm in length which appeared to be coated by a capsule-like sheath. From some filament segments, bacteria have escaped, leaving behind the empty sheath (Supplementary Fig. S3), which collapsed during sample preparation for scanning electron microscopy resulting in a beading phenotype (Supplementary Fig. S2). Interestingly, some of the beads have 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 2xYT broth (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) 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; Ziesché *et al.*, 1985; Heipha Diagnostika) forming small grey colonies after incubation for 48 hours at 27°C. The temperature optimum of strain CN3^T was tested in 2xYT broth, both under aerobic and anaerobic conditions (paraffin overlay) in the range between 16°C and 40°C (Supplementary Fig. S4). After incubation for 18 hours, we found a broad temperature optimum between 20°C

and 30°C and a sharp drop above 36°C and below 20°C, both under aerobic and anaerobic conditions. However, significant growth was also observed at 4°C (incubation in 2xYT broth for 7 days without shaking).

The pH-dependent growth range was tested in buffered 2xYT broth at 22°C 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

The API 20NE test system showed nitrate reduction, glucose fermentation, urease activity and esculin hydrolysis after incubation at 30°C for 48 hours. Neither arginine dihydrolase activity, indole production, gelatine hydrolysis or β-galactosidase activity was observed, nor assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, gluconate, capric acid, adipic acid, malic acid, citrate or phenylacetic acid was shown with the API 20 NE system.

Chemotaxonomic properties

Analysis of respiratory quinones was carried out by the Identification service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany. Major ubiquinone of CN3^T is Q-8. Since Q-8 is also the major ubiquinone of some *Pasteurellaceae* as well as of some *Enterobacteriaceae* and also of other gammaproteobacteria, this result allows no further classification of CN3^T.

Ribotyping applying the Qualicon RiboPrinter system was performed by the Identification service and Dr. Peter Schneider, DSMZ, Braunschweig, Germany. The *Eco*RI RiboPrint pattern showed no significant similarity (>0.85) to entries of the DuPont identification library or to entries of the internal DSMZ database.

The DNA base composition (G + C content) of strain CN3^T was determined by the Identification service and Dr. Peter Schumann, DSMZ, Braunschweig, Germany, 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 G + C content of members of the *Enterobacteriaceae* typically is in the range between 45% to 55% with the exception of *Proteus mirabilis* (38.9%) (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Members of the *Pasteurellaceae* typically have G + C contents 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, Braunschweig, Germany, 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 $C_{18:1}\omega7c$ (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 *Pasteurellaceae* with $C_{18:1}\omega7c$ being no regular constituent (Mutters *et al.*, 1993; Christensen *et al.*, 2007).

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 over night culture of CN3^T in 2xYT or on 2xYT agar plates (27°C), HeLa cells were infected with CN3^T (multiplicity of infection of 50), and infected cultures were further incubated for 3 hours at 37°C in the presence of 5% CO_2. Then the wells were washed five times with PBS (8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KCl, 0.2 g/l KH_2PO_4) and subsequently cells were fixed in methanol for 5 min, stained with Accustain modified Giemsa stain (Sigma) and examined under the microscope. Neither significant adherence of CN3^T to HeLa cells nor any cytopathic effect could be observed. Given the poor growth of CN3^T at 37°C and the lack of

evidence for interaction with HeLa cells we do not expect a pathogenic potential on endotherms. Therefore, isolation from wild boar faeces was likely due to accidental intestinal passage after uptake from soil or feed or due to contamination of faeces that was collected from ground. This is in accordance with our unsuccessful efforts to isolate organisms related to CN3^T from any other faeces sample.

Secreted proteins

We further analysed whether strain CN3^T secretes proteins into the culture supernatant. A single colony was suspended into 3 ml 2xYT broth and cultured for 24 hours 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 bacteria. Subsequently, 1.7 ml of cleared supernatant was transferred to a new tube for precipitation of proteins with 200 µl trichloroacetic acid (TCA). After 1 hour of incubation on ice, samples were centrifuged for 30 minutes at 4°C (14 000 g) and supernatants after centrifugation were discarded. The pellets were washed twice with ice-cold acetone and subsequently air-dried. Pellets were dissolved in 30 µl of SDS-PAGE loading buffer (Trček *et al.*, 2002) and samples of 10 µl 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 (approximately 0.5-2 mg per litre each) irrespective of the presence of oxygen (Supplementary Fig. S5).

Resistance to antibiotics

In a standard microbroth dilution assay according to established protocols (National Committee for Clinical Laboratory Standards, 1997 and 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. 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, respectively, have been determined, reflecting intermediate resistance.

Classification

CN3^T is considered as the representative of a novel species and genus within the *Gammaproteobacteria*. This is substantiated by its isolated phylogenetic position according to 16S rRNA sequence analysis, the presence of the oxidase reaction in contrast to most *Enterobacteriales*, the high relative abundance of fatty acid $C_{18:1}$ ω7c in contrast to *Pasteurellales*, and the distinct ribotype pattern. The name *Orbus hercynius* gen. nov., sp. nov. is proposed. Determination of the exact taxonomic standing within the *Gammaproteobacteria* requires further studies and probably the definition 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 C_{18} ($C_{18:1}$ ω7c) and saturated, even-numbered, straight-chain C_{16} ($C_{16:0}$) 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. 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 and 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 'bulls-eye' appearance on cefsulodin-irgasan-novobiocin (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 nitrate reduction, glucose fermentation, urease activity and esculin hydrolysis. Neither arginine dihydrolase activity, indole production, gelatine hydrolysis or β-galactosidase activity is observed, nor assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, gluconate, capric acid, adipic acid, malic acid, citrate or phenylacetic acid is shown with the API 20 NE system. Major fatty acids are $C_{18:1}$ ω7c, $C_{16:0}$, summed feature 2 (iso-$C_{16:1}$ I, $C_{14:0}$ 3-OH and/or $C_{12:0}$ aldehyde), summed feature 3 ($C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH) and $C_{14:0}$. Major ubiquinone is Q-8. The G + C content of the DNA of the type strain of the species is 36.4 mol%. The type strain, CN3^T (=DSM 22228^T=CCUG 57622^T), was isolated from faeces of wild boar collected at the zoo of Halberstadt (Germany).

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246 Robert Koch-Institute in Berlin.

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LEGENDS TO FIGURES

Fig. 1. Neighbour-joining phylogenetic reconstruction from nearly 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* into the analysis. Scale bar indicates 1 nucleotide substitution per 100 nucleotides.

Fig. 2. (A) Scanning electron photomicrograph of strain CN3^T after cultivation on 2xYT agar for 40 hours at 27°C. (B) Transmission electron micrograph (negative staining) of a phage of strain CN3^T.

FOOTNOTES

The 16S rRNA partial DNA sequence was deposited at GenBank under accession no. FJ612598. Strain CN3^T was deposited at the DSMZ strain collection (DSM 22 228^T) and at the Culture Collection of the University of Göteborg (CCUG 57622^T). Supplementary material is available in IJSEM Online.

TABLES

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
$C_{16:1}$ ω5c	0.17
$C_{16:0}$	33.73
$C_{18:1}$ ω7c	38.45
$C_{18:0}$	0.35
Summed feature 2*	9.37
Summed feature 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 $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH

Fig. 1

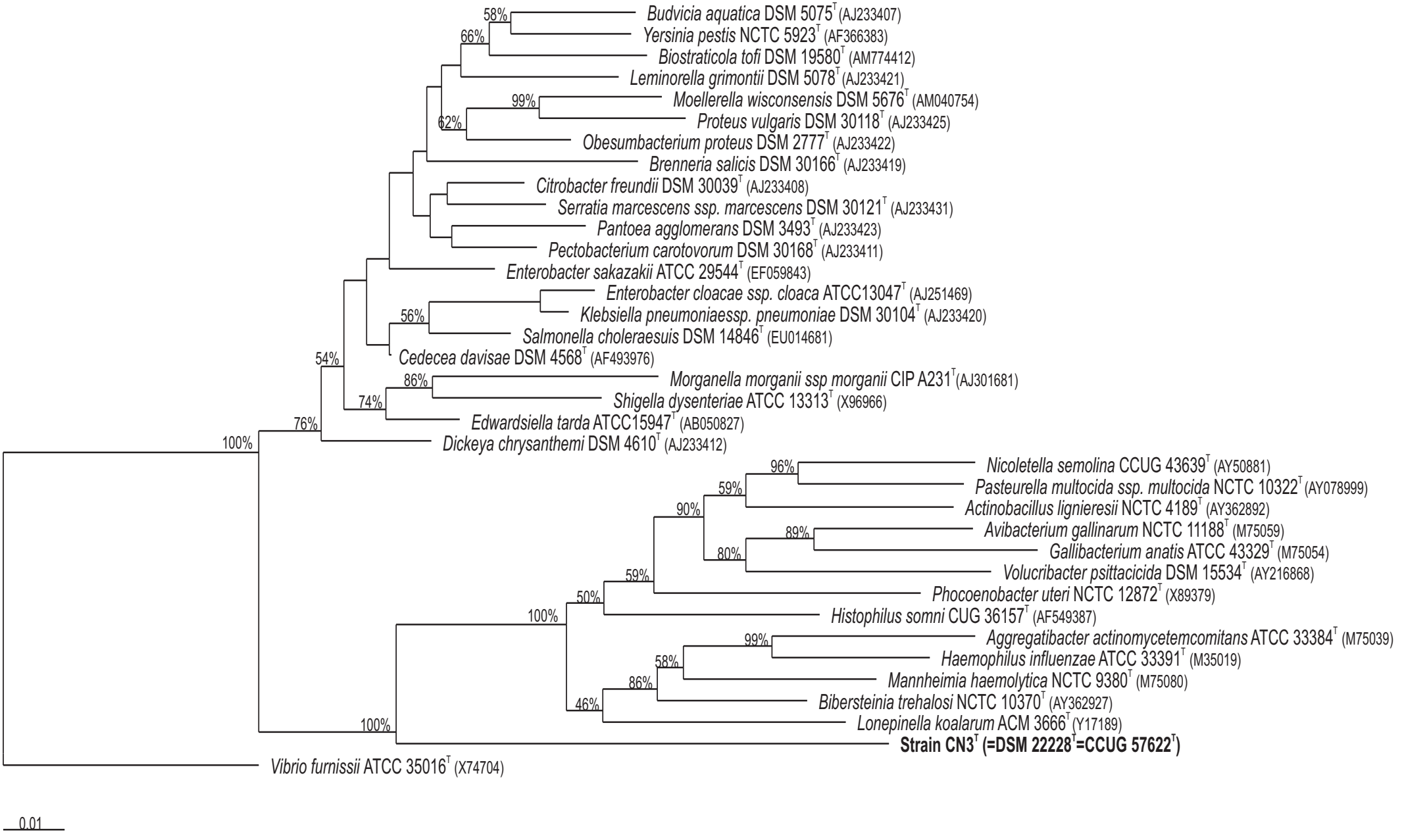
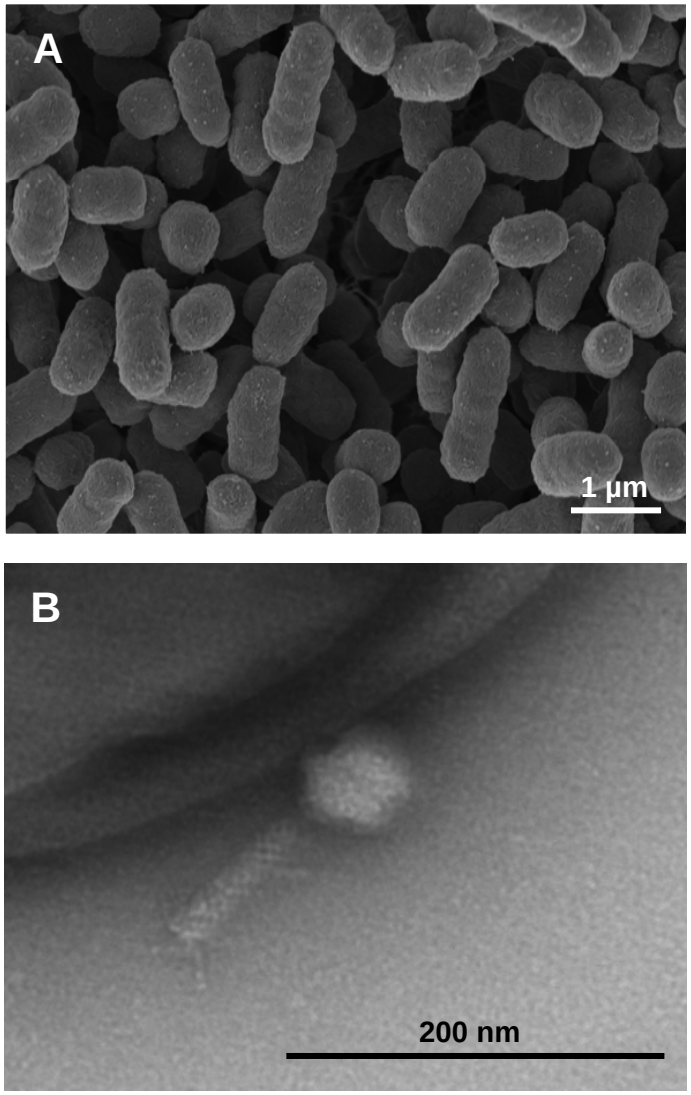
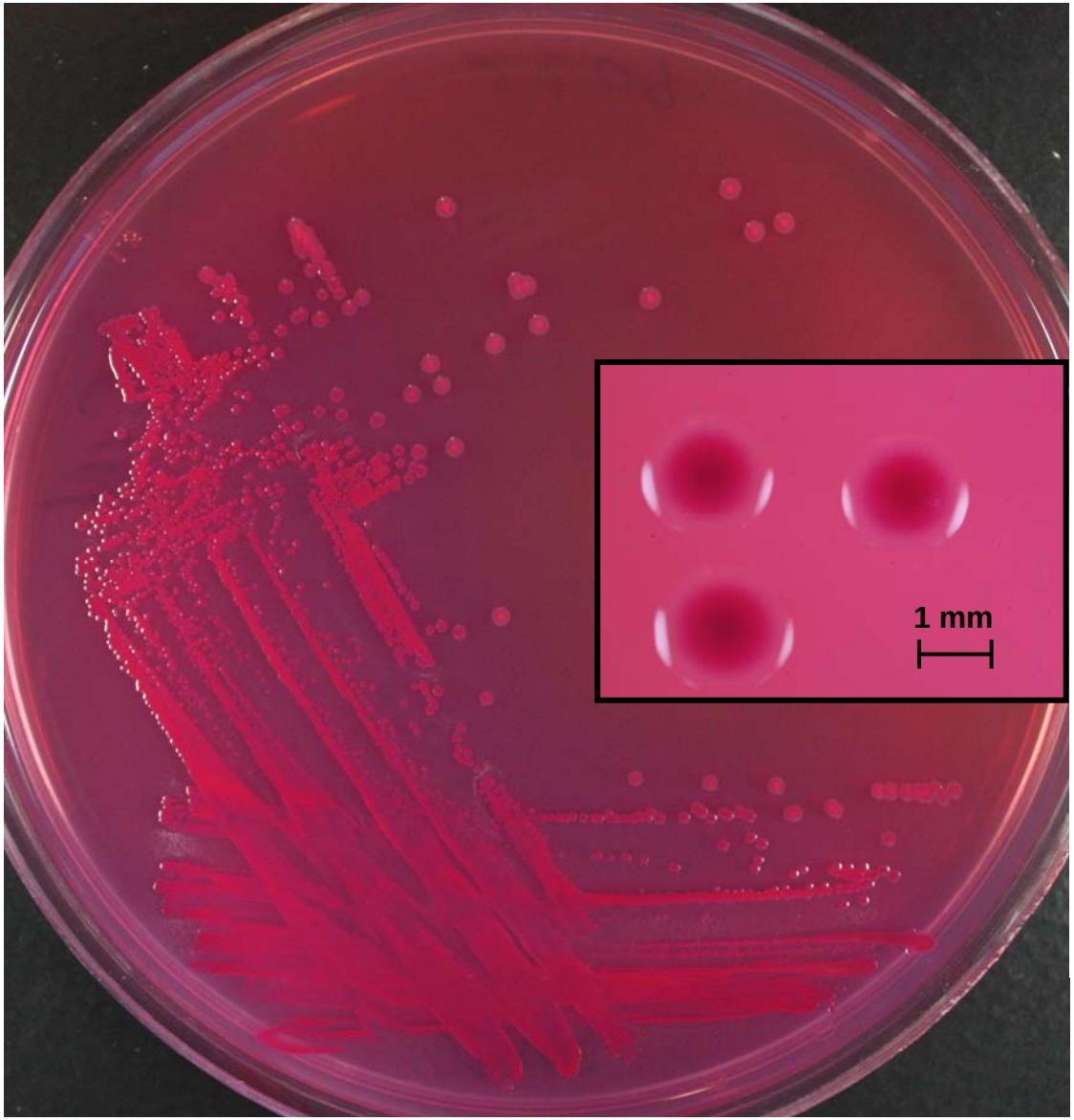

58%
66%
99%
62%
54%
56%
86%
74%
76%
100%
96%
59%
90%
89%
80%
59%
50%
100%
99%
58%
86%
46%
100%
Budvicia aquatica DSM 5075T (AJ233407)
Yersinia pestis NCTC 5923T (AF366383)
Biostraticola tofi DSM 19580T (AM774412)
Leminorella grimontii DSM 5078T (AJ233421)
Moellerella wisconsensis DSM 5676T (AM040754)
Proteus vulgaris DSM 30118T (AJ233425)
Obesumbacterium proteus DSM 2777T (AJ233422)
Brenneria salicis DSM 30166T (AJ233419)
Citrobacter freundii DSM 30039T (AJ233408)
Serratia marcescens ssp. marcescens DSM 30121T (AJ233431)
Pantoea agglomerans DSM 3493T (AJ233423)
Pectobacterium carotovorum DSM 30168T (AJ233411)
Enterobacter sakazakii ATCC 29544T (EF059843)
Enterobacter cloacae ssp. cloaca ATCC13047T (AJ251469)
Klebsiella pneumoniaessp. pneumoniae DSM 30104T (AJ233420)
Salmonella choleraesuis DSM 14846T (EU014681)
Cedecea davisae DSM 4568T (AF493976)
Morganella morganii ssp morganii CIP A231T(AJ301681)
Shigella dysenteriae ATCC 13313T (X96966)
Edwardsiella tarda ATCC15947T (AB050827)
Dickeya chrysanthemi DSM 4610T (AJ233412)
Nicoletella semolina CCUG 43639T (AY50881)
Pasteurella multocida ssp. multocida NCTC 10322T (AY078999)
Actinobacillus lignieresii NCTC 4189T (AY362892)
Avibacterium gallinarum NCTC 11188T (M75059)
Gallibacterium anatis ATCC 43329T (M75054)
Volucribacter psittacicida DSM 15534T (AY216868)
Phocoenobacter uteri NCTC 12872T (X89379)
Histophilus somni CUG 36157T (AF549387)
Aggregatibacter actinomycetemcomitans ATCC 33384T (M75039)
Haemophilus influenzae ATCC 33391T (M35019)
Mannheimia haemolytica NCTC 9380T (M75080)
Bibersteinia trehalosi NCTC 10370T (AY362927)
Lonepinella koalarum ACM 3666T (Y17189)
Strain CN3T (=DSM 22228T=CCUG 57622T)
Vibrio furnissii ATCC 35016T (X74704)
0.01

Fig. 2

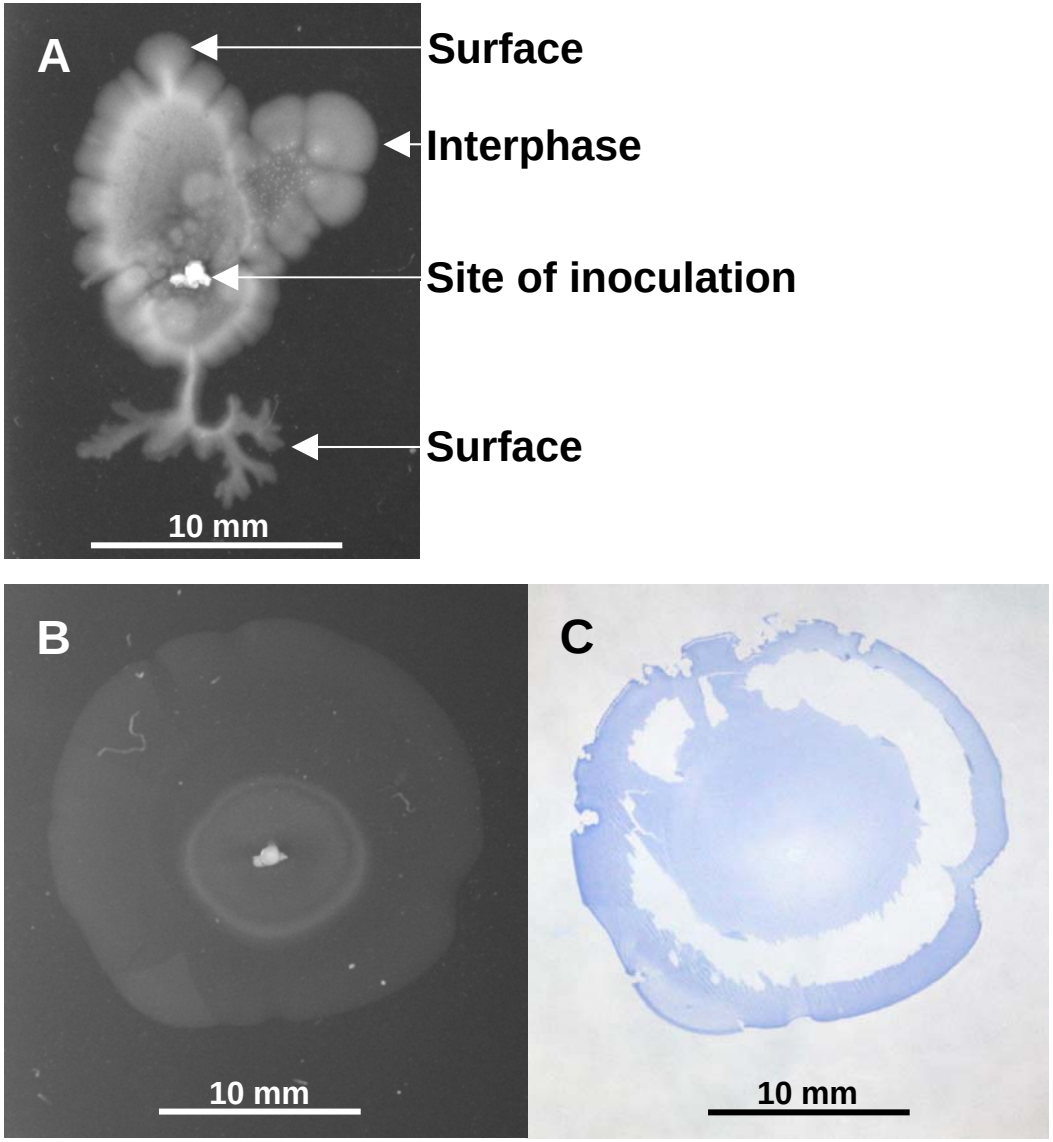


Supplementary Fig. S1



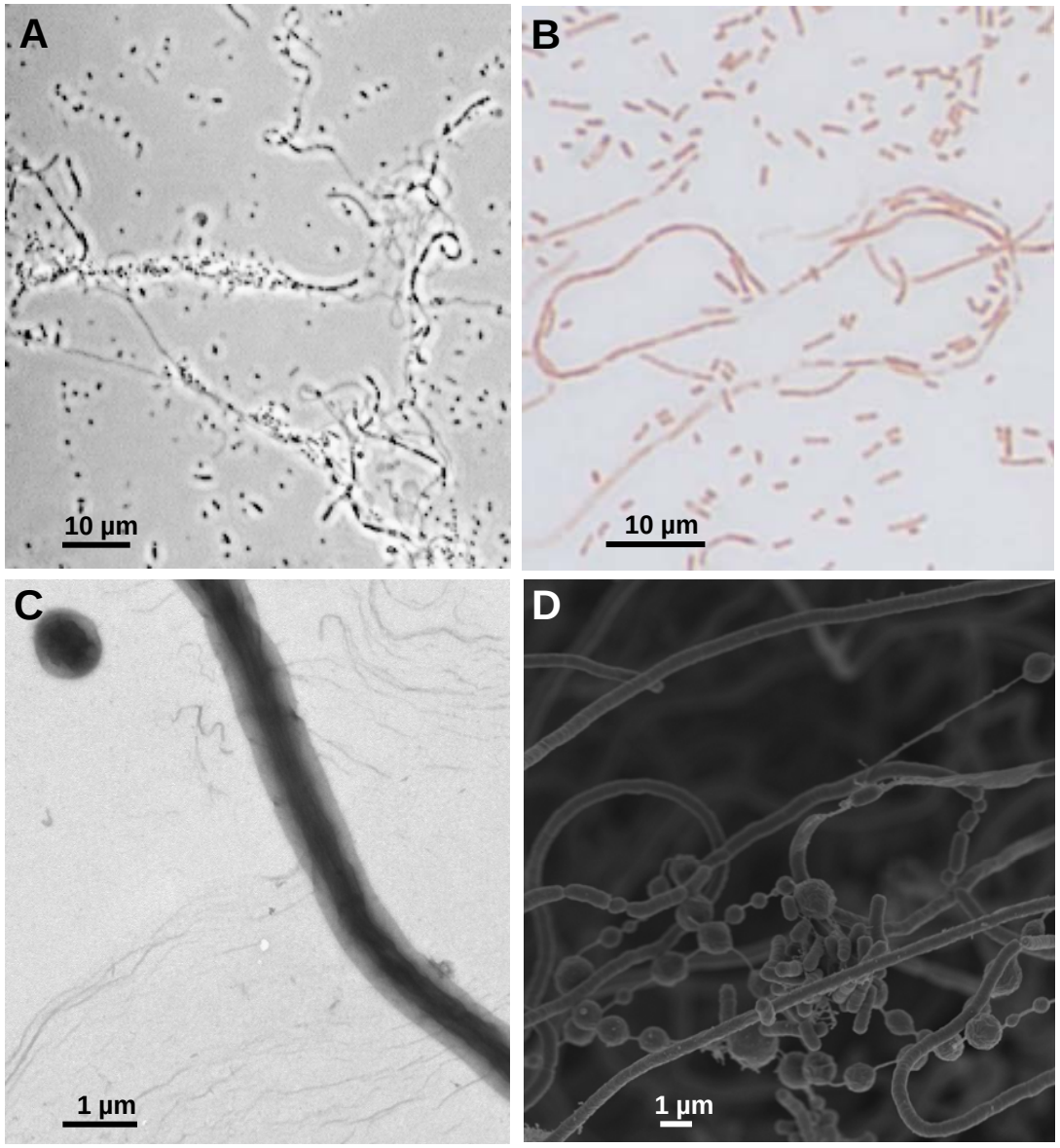
Growth of strain CN3^T on cefsulodin-irgasan-novobiocin (CIN) agar after incubation for 40 hours at 27°C. Inset with colonies at higher magnification; scale as indicated.

Supplementary Fig. S2



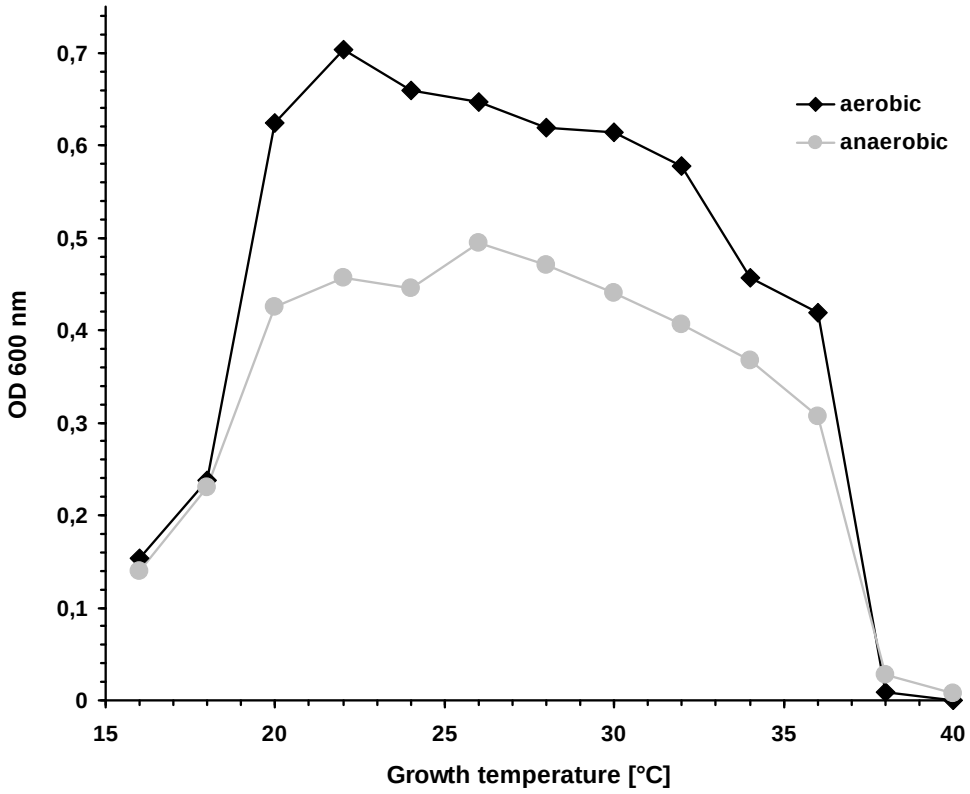
(A) Motility of strain CN3^T on 0.3% agar (2xYT); incubation for 7 days at 27°C. Growth occurs on the surface of the semi-solid agar and at the interphase between Petri-dish and agar. For inoculation the agar was completely punctured at the site indicated. (B) Motility of strain CN3^T at the interphase between Petri-dish and a 1% agarose layer containing 2xYT broth; incubation for 7 days at 27°C. (C) The formation of a biofilm at the interphase of the plate shown in (B) was visualized by Coomassie-staining of bacterial mass sticking to the Petri-dish after removal of the agar.

Supplementary Fig. S3



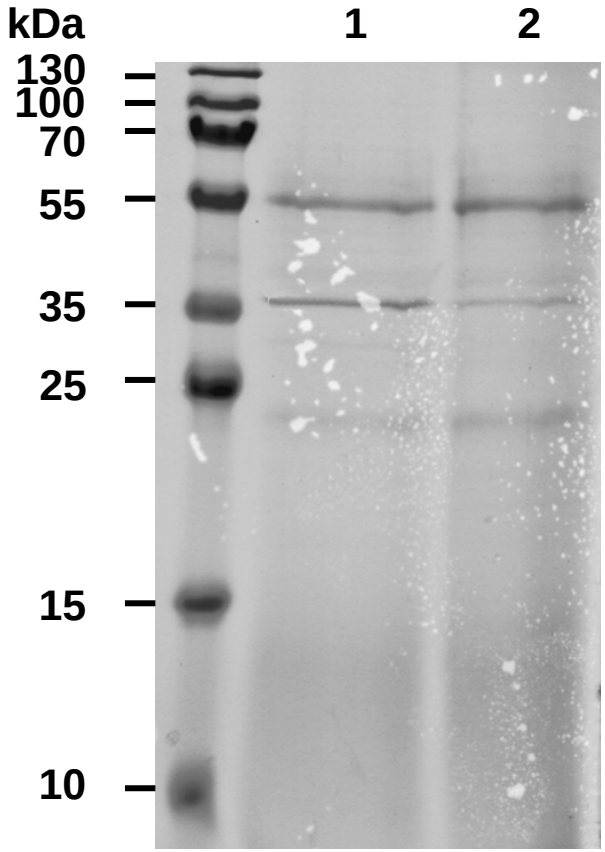
(A) Phase contrast microscopy of the filamentous mutant of strain CN3^T.
(B) Gram staining of the filamentous mutant of strain CN3^T.
(C) Transmission electron micrograph of the filamentous mutant of strain CN3^T.
(D) Scanning electron photomicrograph of the filamentous mutant of strain CN3^T.

Supplementary Fig. S4



Temperature-dependence of growth of strain CN3^T in 2xYT broth under aerobic and anaerobic conditions. Each data point represents three independent cultures.

Supplementary Fig. S5



Coomassie-stained denaturing SDS-PAGE analysis of secreted proteins of strain CN3^T. Supernatant from a culture of strain CN3^T grown in 2xYT broth for 24 hours under aerobic (lane 1) or anaerobic (lane 2) conditions was precipitated with trichloroacetic acid and subjected to denaturing gel electrophoresis. Samples loaded correspond to supernatant from approx. 600 µl of bacterial culture. Protein ladder (PageRuler Plus Prestained) was purchased from Fermentas.