Cloacibacterium normanense gen. nov., sp. nov., a novel bacterium in the family *Flavobacteriaceae* isolated from municipal wastewater

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Phenotypic and phylogenetic studies were performed on three isolates of an unknown Gram-negative, facultatively anaerobic, non-motile, yellow-pigmented, rod-shaped organism isolated from raw sewage. 16S rRNA gene sequence analysis indicated that these strains were members of the *Bergeyella*–*Chryseobacterium*–*Riemerella* branch of the family *Flavobacteriaceae*. The unknown bacterium was readily distinguished from reference strains by 16S rRNA gene sequencing and biochemical tests. The organism contained menaquinone MK-6 as the predominant respiratory quinone and had a DNA G + C content of 31 mol%. A most probable number-PCR approach was developed to detect, and estimate the numbers of, this organism. Untreated wastewater from one plant yielded an estimated count of 1.4×10^5 cells ml⁻¹, and untreated wastewater from a second plant yielded an estimated count of 1.4×10^4 cells ml⁻¹. Signal was not detected from treated effluent or from human stool specimens. On the basis of the results of the study presented, it is proposed that the unknown bacterium be classified in a novel genus *Cloacibacterium*, as *Cloacibacterium normanense* gen. nov., sp. nov., which is also the type species. The type strain of *Cloacibacterium normanense* is strain NRS1^T (=CCUG 46293^T = CIP 108613^T = ATCC BAA-825^T = DSM 15886^T).

Members of the family *Flavobacteriaceae* are ubiquitous in aquatic habitats, where they are generally thought to play a role in the breakdown of complex organic matter (Bernardet *et al.*, 2002). Members of this group are common in activated sludge and other parts of wastewater-treatment plants (Benedict & Carlson, 1971; Güde, 1980). By using probes specific for the *Cytophaga*–*Flavobacterium* group, researchers have found that this group constitutes a significant portion (11–24 %) of the microbial community of activated sludge from wastewater plants that employ enhanced biological phosphate removal (Wagner *et al.*, 1994; Liu *et al.*, 2005), and that its members contribute directly to phosphate removal in activated sludge (Van Ommen Kloeke & Geesey, 1999). Three isolates of a numerically dominant, heterotrophic, Gram-negative bacterium were recovered during a study of the impact of the discharge of treated wastewater into the Canadian River at Norman, OK, USA. Biochemical and phenotypic analysis showed that the organisms

possessed traits similar to those of species belonging to the *Cytophaga*–*Flavobacterium*–*Bacteroides* group of organisms (Paster *et al.*, 1985; Segers *et al.*, 1993). Studies involving 16S rRNA gene sequence analysis indicated that the isolates represented a new subline within the *Bergeyella*–*Chryseobacterium*–*Riemerella* branch of the *Flavobacteriaceae* (Vandamme *et al.*, 1994; Bernardet *et al.*, 1996). A most probable number (MPN)-PCR approach was developed to detect (and enumerate) this organism from the wastewater influent and from human stool samples, a possible source of the bacterium. It is therefore proposed that a novel genus and species be created to accommodate the novel organism recovered from sewage.

Isolates NRS1^T, NRS30 and NRS32 were isolated from untreated wastewater from a water-treatment plant located at Norman, OK, USA. Strain NRS1^T was isolated as a most numerous culturable heterotroph from an MPN dilution series in 0.5 × tryptic soy broth (Becton Dickinson). Strains NRS30 and NRS32 were isolated by direct plating of untreated wastewater from the same source on nutrient agar. Tryptic soy broth or tryptic soy agar (Becton Dickinson) was used for routine culture unless otherwise

Abbreviation: MPN, most probable number.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CCUG 46293^T is AJ575430.

stated. The strains were characterized biochemically by using a combination of conventional tests performed as described previously (Smibert & Krieg, 1991), and by using the API Rapid ID 32S, API Rapid ID 32A and API ZYM test systems according to the manufacturer's instructions (bioMérieux). Hydrolysis of casein, DNA and urea and reduction of nitrate were assessed by inoculation on the appropriate media (Becton Dickinson). Citrate utilization was assessed by using Simmons' citrate agar (Becton Dickinson). Hydrolysis of xanthine, hypoxanthine and uric acid was investigated by using the method of Bowman *et al.* (1996). Hydrolysis of alginate and chitin was investigated by using the method of West & Colwell (1984) and pectin hydrolysis was assessed by using the method of Hildebrand (1971). Resistance to antibiotics was investigated by using the method of Bauer *et al.* (1966). The fermentation products of glucose were determined by using ion-exclusion HPLC with an Aminex HPX-87H column (Bio-Rad) and a mobile phase consisting of 0.0025 M H₂SO₄. The pH range and optimum was determined by incubation on 1.5% tryptone broth supplemented with one of the following (at 30 g l⁻¹): HOMOPIPES [homopiperazine-*N,N'*-bis-2-(ethanesulfonic acid)] at pH 4 and 5, MES [2-(*N*-morpholino)ethanesulfonic acid] at pH 6, TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] at pH 7 and 8 or CAPSO [3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid] at pH 9.

Fatty acid methyl ester analysis was performed on overnight cultures grown on tryptic soy agar at 37 °C by Microcheck, Inc. (Northfield, VT, USA) according to methods described previously (Miller, 1982; Sassar, 1990). For the detection of flexirubin-type pigments, pigmented cells were subjected to the KOH test as previously described for preliminary analysis (Hirsch & Reichenbach, 1981). For further characterization of pigments, cells were grown on tryptic soy agar and the pigments were extracted with acetone. The cell debris was then pelleted and the crude extract was analysed by using a DU 640 spectrophotometer (Beckman Coulter). Isoprenoid quinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC as described by Groth *et al.* (1997). DNA was isolated by the method of Lawson *et al.* (1989). The DNA G + C content (mol%) was determined by HPLC according to Mesbah *et al.* (1989). The 16S rRNA genes of the isolates were amplified by PCRs using universal primers pA (positions 8–28, *Escherichia coli* numbering) and pH (positions 1542–1522) (Hutson *et al.*, 1993). The amplified products were purified by using a QIAquick PCR purification kit (Qiagen) and directly sequenced with primers directed towards conserved positions of the rRNA gene and the dRhodamine terminator cycle sequencing kit (ABI) using an automatic DNA sequencer. The closest known relatives of the novel isolates were determined by performing database searches using the program FASTA (Pearson & Lipman, 1985). These sequences and those of other related strains were retrieved from GenBank and aligned with the newly determined sequences by using the program SEQtools (Rasmussen, 2002). The resulting

multiple sequence alignment was corrected manually using the program GeneDoc (Nicholas *et al.*, 1997) and a phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the programs SEQtools and TREEVIEW (Page, 1996). The stability of the groupings was estimated by bootstrap analysis (1000 replications) using the same programs. For the MPN-PCR analysis, a pair of oligonucleotide primers, Cloac-001f (5'-TATTGTTTCTTCGGAATGA) and Cloac-001r (5'-ATGGCAGTTCTATCGTTAAGC), were designed (using Primer3 software; Rozen & Skaletsky, 2000) that were specific to the 16S rRNA gene of the newly isolated organism. DNA was isolated from 10 human stool samples (200 mg each) by using the QIAamp DNA Stool Mini kit (Qiagen) according to the manufacturer's instructions. Cells were harvested from wastewater influent and effluent samples by centrifugation and were then washed with a saline buffer. Washed whole cells were then added to the PCR mixture after a freeze-thaw cycle to achieve lysis. MPN-PCR (Picard *et al.*, 1992) was performed by means of serial 10-fold dilutions in triplicate and visualization of the PCR product by agarose gel electrophoresis. The MPN was calculated according to the tables of Cochran (1950).

All strains stained Gram-negative and displayed pleomorphic cell morphologies depending on the medium used. In broth cultures, most cells were long (up to 27 µm); when cultured on agar plates, most cells were much shorter (5–9 µm). Similar pleomorphic cell morphologies have been described for members of the *Flavobacteriaceae* (Simon & White, 1971). A battery of phenotypic and biochemical tests, including API Rapid ID 32S, API Rapid ID 32A and API ZYM, were performed on all strains, the results of which appear in the genus and species descriptions and in Table 1. Sensitivities to the following antibiotics were tested: ampicillin (10 µg), carbenicillin (100 µg), cefaclor (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), oxytetracycline (30 µg), streptomycin (10 µg), sulfathiazole (0.25 mg), tetracycline (30 µg) and trimethoprim (5 µg). All three strains were resistant to erythromycin and kanamycin; strain NRS1^T was also resistant to streptomycin and strain NRS32 was resistant to nalidixic acid. The KOH test for flexirubin-type pigments was negative for all strains. The absorbance spectra of crude pigment extract from all strains exhibited a triple-peak signature (418, 451 and 483 nm) characteristic of carotenoid-type pigments (Schmidt *et al.*, 1994). The fatty acid profiles of the newly isolated strains consisted mainly of branched-chain fatty acids, with iso-C_{13:0} (8–9%), iso-C_{15:0} (40–46%), iso-C_{15:1} (7–10%) and iso-C_{17:0} 3-OH (5–9%) predominating. The detailed fatty acid composition is shown in Table 2.

To ascertain the phylogenetic relationships of the unknown isolates, their 16S rRNA genes were sequenced and subjected to a comparative analysis. The almost complete gene sequences (> 1500 nt) of the three strains were determined;

Table 1. Differentiating characteristics for *Cloacibacterium* and other related genera

Symbols: +, positive for all strains studied; –, negative for all strains studied; v, varies within or between strains and species; ND, not determined for most strains. Data for *Bergeyella* and *Chryseobacterium* were obtained from Vandamme *et al.* (1994), Shimomura *et al.* (2005) and de Beer *et al.* (2005) and data for *Riemerella* were obtained from Segers *et al.* (1993) or from Vancanneyt *et al.* (1999).

Characteristic	<i>Cloacibacterium</i>	<i>Bergeyella</i>	<i>Riemerella</i>	<i>Chryseobacterium</i>
Capnophilic metabolism	–	–	+	–
Production of:				
Yellow pigment	+	–	–	+
DNase	+	–	ND	+
Urease	–	+	v	v
Indole	+	+	v	v
Acid from glucose	+	–	+	v
Aesculin hydrolysis	+	–	v	+
Growth at:				
37 °C	+	+	+	v
42 °C	–	–†	+	–‡
DNA G + C content (mol%)	31	35–37	33–37	33–38
Habitat	Free-living	Parasitic or saprophytic	Parasitic	Free-living or parasitic

*Positive for most strains studied.

†Positive for one of the 30 *B. zoohelcum* strains studied.

‡Negative for most strains studied.

pairwise analysis showed that strain NRS1^T exhibited 100 % 16S gene sequence similarity to strain NRS30 and 99.6 % similarity to strain NRS32. Sequence database searches confirmed that the unidentified, rod-shaped organism from human raw sewage did not correspond to any recognized Gram-negative bacterium and represented a previously

unknown taxon. The results of neighbour-joining analysis, shown in Fig. 1, confirmed that the unknown bacterium (as exemplified by strain NRS1^T) is a member of the *Bergeyella*–*Chryseobacterium*–*Riemerella* cluster of the *Flavobacteriaceae*. In particular, the unknown bacterium forms an association with a cluster of organisms that includes *Riemerella anatipestifer* (88.8 % sequence similarity), *Riemerella columbina* (94.6 % sequence similarity), *Bergeyella zoohelcum* (94.4 % sequence similarity) and an as-yet uncultured bacterium (99.2 % sequence similarity) recovered from uranium mining waste (Fig. 1).

Because this organism is present in large numbers in untreated wastewater, it was postulated that the source of the organism may have been the human gastrointestinal tract. An MPN-PCR strategy was developed to investigate this possibility. Primers were designed that were specific to the 16S rRNA gene sequence of the newly isolated organism, yielding a 557 bp PCR fragment from DNA isolated from all three strains. Organisms phylogenetically close to the three isolates (*Bergeyella* and *Riemerella* species) were challenged with the primers and gave negative results (data not shown). A pure culture of strain NRS1^T yielded counts of 1.44×10^7 cells ml^{–1} from MPN-PCR and the spread-plate method yielded a mean cell count of 4.03×10^7 cells ml^{–1}, showing that comparable numbers of organisms can be recovered by these two methods. Untreated wastewater from the Norman plant yielded an estimated count of 1.38×10^5 cells ml^{–1} and wastewater from another plant (Moore, OK, USA) yielded an estimated 1.38×10^4 cells ml^{–1}. Signal was not detected from wastewater effluent. Signal was also not detected in any of the 10 human

Table 2. Cellular fatty acid composition of *Cloacibacterium* strains

Values are percentages of total fatty acids. –, Not detected or present at less than 1 %.

Fatty acid	NRS1 ^T	NRS30	NRS32
C _{12:0} 3-OH	2.1	1.0	2.1
iso-C _{13:0}	7.9	9.2	9.2
anteiso-C _{13:0}	1.1	–	1.0
iso-C _{14:0}	3.3	2.8	3.4
C _{15:0}	1.1	–	–
iso-C _{15:0}	40.8	43.0	45.8
anteiso-C _{15:0}	5.0	5.7	6.2
iso-C _{15:0} 3-OH	3.5	4.4	3.9
iso-C _{15:1}	7.8	7.0	10.2
anteiso-C _{15:1}	5.4	4.0	–
C _{16:0}	–	1.3	–
iso-C _{16:0}	1.7	–	1.0
C _{16:0} 3-OH	1.0	–	–
iso-C _{16:0} 3-OH	2.5	1.9	1.8
C _{16:1} ω5c	1.0	–	–
iso-C _{17:0} 3-OH	5.3	8.6	4.8

Fig. 1. Unrooted tree showing the phylogenetic relationships of *Cloacibacterium normanense* gen. nov., sp. nov. and some related Gram-negative bacteria. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1327 nt. Bootstrap values, expressed as percentages of 500 replications, are given at branching points. Bar, 1% sequence divergence.

stool samples, indicating that the newly isolated bacterium was not a dominant member of the human gastrointestinal tract (though it cannot be discounted as being present in smaller numbers, below the level of detection of the MPN-PCR method used). More samples may need to be screened to confirm this result. However, this organism was found in large numbers in untreated wastewater, where it may play a role in the removal of phosphate. A phylogenetic relative of this organism (*Chryseobacterium defluvii*) was isolated from a phosphate-removing mixed culture obtained from activated sludge (Kämpfer *et al.*, 2003).

On the basis of tree-topology considerations and sequence-divergence values of 5% or more with respect to the aforementioned taxa, the unidentified bacterium was only distantly related to these taxa and merits classification at a similar taxonomic rank (i.e. genus). Therefore, on the basis of both the phenotypic and the phylogenetic findings, we consider that the unknown Gram-negative rod isolated from raw sewage merits classification in a novel genus and a novel species, for which the name *Cloacibacterium normanense* gen. nov., sp. nov. is proposed. Characteristics that are useful in distinguishing *Cloacibacterium normanense* from its closest phylogenetic relatives are shown in Table 1.

Description of *Cloacibacterium* gen. nov.

Cloacibacterium (Clo.a'ci.bac.te'ri.um. L. fem. n. *cloaca* a sewer, canal; L. neut. n. *bacterium* a small rod; N.L. neut. n. *Cloacibacterium* a sewer rod).

Cells are Gram-negative, non-motile and pleomorphic rod-shaped. Pigment is not of the flexirubin-type. Yellow to orange carotenoid-type pigments are produced. Facultatively anaerobic. Catalase- and oxidase-positive. The major end product of glucose fermentation under both aerobic and anaerobic conditions is pyruvate. Fatty acids consist mainly of branched-chain fatty acids, with iso-C_{13:0}, iso-C_{15:0}, iso-C_{15:1} and iso-C_{17:0} 3-OH predominating. The predominant respiratory quinone is MK-6. The DNA G + C content of the type strain of the type species is 31 mol%. The type species is *Cloacibacterium normanense*.

Description of *Cloacibacterium normanense* sp. nov.

Cloacibacterium normanense (nor.man.en'se. N.L. neut. adj. *normanense* pertaining to the city of Norman, OK, USA, where the organism was first isolated).

Displays the following features in addition to those given in the genus description. In broth cultures, cells are long (up to 27 µm); cells from agar plate cultures are much shorter (5–9 µm). After 48 h, colonies grown on tryptic soy agar are 1.0–1.5 mm in diameter, round, entire and very waxy. Growth occurs between 18 and 36 °C. No growth occurs at 4 °C or at 40 °C or above. No growth occurs on MacConkey agar. Optimum temperature for growth is 30 °C. All strains grow at pH 7 and 8; some strains also grow at pH 6 and 9. The pH optimum for all strains is 7. Indole-positive. Starch, aesculin, gelatin and casein are hydrolysed and DNA is hydrolysed weakly. Urea, chitin, pectin, alginate, uric acid,

xanthine and hypoxanthine are not hydrolysed. Methyl red- and Voges–Proskauer-negative. Nitrate is not reduced. Cellulose and agar are not degraded. Most isolates tested are resistant to erythromycin (15 µg) and kanamycin (30 µg). All isolates tested are sensitive to carbenicillin (100 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), gentamicin (10 µg), oxytetracycline (30 µg), sulfathiazole (0.25 mg) and tetracycline (30 µg). With the API kits, acid is produced with α -cyclodextrin and mannose. Acid is not produced from alanine, ribose, mannitol, sorbitol, lactose, trehalose, raffinose, hippurate, glycogen, melibiose, melezitose, sucrose, L-arabinose, D-arabitol, tagatose or raffinose. Enzyme activity is detected for alkaline phosphatase, acid phosphatase, alanine arylamidase, arginine dihydrolase (weak reaction), arginine arylamidase, chymotrypsin, esterase C-4 (weak reaction), ester lipase C8, α -glucosidase, β -glucosidase, glycine arylamidase, glycyl tryptophan arylamidase, proline arylamidase, leucine arylamidase, leucyl glycine arylamidase, maltose, phenylalanine arylamidase, alanine phenylalanine proline arylamidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, pyroglutamic acid arylamidase, tyrosine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, serine arylamidase and valine arylamidase. Enzyme activity is not detected for *N*-acetyl- β -glucosaminidase, α -arabinosidase, α -fucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, glutamic acid arylamidase, α -mannosidase, β -mannosidase, methyl β -D-glucopyranoside, pyroglutamic acid arylamidase, lipase C14 or trypsin. Fatty acids iso-C_{13:0} (8–9 %), iso-C_{15:0} (40–46 %), iso-C_{15:1} (7–10 %) and iso-C_{17:0} 3-OH (5–9 %) predominate. The DNA G + C content is 31 mol%.

The type strain, NRS1^T (=CCUG 46293^T = CIP 108613^T = ATCC BAA-825^T = DSM 15886^T), was isolated from untreated human wastewater. Additional strains of the species, strains NRS30 (=CCUG 48043) and NRS32 (=CCUG 48044), were also isolated from wastewater.

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