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Ignavigranum ruoffiae sp. nov., isolated from human clinical specimens

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Two strains of a hitherto undescribed Gram-positive catalase-negative, facultatively anaerobic coccus isolated from human sources were characterized by phenotypic and molecular taxonomic methods. Comparative 16S rRNA gene sequencing studies demonstrated the unknown strains were genealogically identical, and constitute a new line close to, but distinct from, the genera *Facklamia* and *Globicatella*. The unknown bacterium was readily distinguished from *Facklamia* species and *Globicatella sanguinus* by biochemical tests and electrophoretic analysis of whole-cell proteins. Based on phylogenetic and phenotypic evidence it is proposed that the unknown bacterium be classified as *Ignavigranum ruoffiae* gen. nov., sp. nov. The type strain of *Ignavigranum ruoffiae* is CCUG 37658^T.

Keywords: *Ignavigranum ruoffiae*, taxonomy, phylogeny, 16S rRNA

During the past decade, knowledge of the taxonomic interrelationships of the Gram-positive catalase-negative cocci has improved markedly. Much of this improvement has resulted from using a range of phenotypic methods (e.g. miniaturized biochemical testing, protein profiling) in concert with molecular genetic approaches, notably comparative 16S rRNA gene sequencing. 16S rRNA gene sequence analysis has not only facilitated new insights into the phylogenetic interrelationships of the Gram-positive catalase-negative cocci but has provided systematists with an immensely powerful means for characterizing new diversity. Indeed primarily as a result of an increasing recognition by clinical microbiologists of the possible role of these organisms as opportunistic human pathogens, in conjunction with the use of this molecular taxonomic tool, a plethora of new species and genera of Gram-positive catalase-negative cocci [e.g. *Aerococcus urinae* (Aguirre & Collins, 1992b), '*Abiotrophia elegans*' (Roggenkamp *et al.*, 1998), *Alloiococcus* (Aguirre & Collins, 1992a), *Dolosigranulum* (Aguirre *et al.*, 1993), *Facklamia* (Collins *et al.*, 1997), *Globicatella* (Collins *et al.*, 1992) and *Helcococcus* (Collins *et al.*, 1993)] have been discovered and described in recent years. In this study we have used 16S rRNA gene sequencing to phylogenetically characterize two strains of a hitherto unknown *Abiotrophia*-like bacterium from human clinical specimens. Based on the results of a polyphasic taxonomic study, a new genus and species, *Ignavigranum ruoffiae*, is described.

Two strains (1607-97 and 3955-95) from human sources were referred to the Centers for Disease Control and Prevention (Atlanta, GA, USA) for identification. Strain 1607-97 was recovered from a wound infection and strain 3955-95 was isolated from an ear abscess. Both strains have been deposited in the Culture Collection of the University of Göteborg (CCUG), Sweden, under accession numbers CCUG 37658^T and CCUG 37841, respectively. Strains were cultured on Columbia agar (Difco) supplemented with 5% (v/v) horse blood at 37 °C, in air plus 5% (v/v) CO_2. The strains were biochemically characterized by using the API Rapid ID32 Strep, API CORYNE and API ZYM systems according to the manufacturer's instructions (API bioMérieux). The strains were also examined using conventional biochemical tests as described by Facklam & Elliot (1995). PAGE of whole-cell proteins was performed as described by Pot *et al.* (1994). The Pharmacia-LKB UltroScan XL densitometer, with its software, was used for capture of gel data. For normalization and interpretation of protein patterns the GelCompar GCW 3.0 software package (Applied Maths) was used. The cell-wall murein structure and mol% G+C content of DNA of strain CCUG 37658^T were determined by methods described by Schleifer & Kandler (1972) and Garvie (1978),

The GenBank accession number for the 16S rRNA sequence of CCUG 37658^T is Y16426.

respectively. The 16S rRNA genes of the isolates were amplified by PCR and directly sequenced using a *Taq* Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the new isolates were determined by performing database searches. These sequences and those of other known related strains were retrieved from GenBank or Ribosomal Database Project (RDP) databases and aligned with the newly determined sequences using the program PILEUP (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated using the programs PRETTY and DNADIST (using the Kimura-2 correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989). In addition a parsimony analysis (Felsenstein, 1989) was also performed on the same data set.

The two clinical isolates were ovoid in shape and formed single cells, pairs or groups. The strains were Gram-positive, non-spore-forming, catalase-negative, oxidase-negative facultative anaerobes. Both CCUG 37658^T and CCUG 37841 grew in 6·5% NaCl and weakly at 45 °C (growth apparent only after 7 d incubation). The only positive conventional tests other than growth at 6·5% NaCl and at 45°C were reactions for PYRase and LAPase by the disc method. Using the API systems the isolates produced acid from glucose and were similar to each other in not producing acid from D-arabitol, L-arabinose, cyclodextrin, glycogen, lactose, melibiose, melezitose, methyl-β-D-glucopyranoside, pullulan, raffinose, ribose, sorbitol, tagatose, trehalose or D-xylose. Strain CCUG 37841 showed weak acid production from mannitol and sucrose. Acid production from mannitol and sucrose was either negative or weak for strain CCUG 37658^T. Using the API systems the two isolates showed arginine dihydrolase, leucine arylamidase, pyroglutamic acid arylamidase and urease activity; no activity was detected for alkaline phosphatase, alanyl-phenylalanine-proline arylamidase, *N*-acetyl-glucosaminidase, cystine arylamidase, chymotrypsin, α-fucosidase, α-galactosidase, β-galactosidase, β-galacuronidase, α-glucosidase, β-glucosidase, β-glucuronidase, glycyl-tryptophan arylamidase, lipase C14, β-mannosidase, pyrazinamidase, trypsin or valine arylamidase. Neither of the isolates hydrolysed aesculin, hippurate or gelatin. They were Voges–Proskauer-negative and did not reduce nitrate. The close phenotypic affinity between the clinical isolates was confirmed by PAGE analysis of whole-cell proteins in which the two strains formed a robust and tight cluster which was quite separate from all other Gram-positive catalase-negative reference organisms examined, including, *Abiotrophia* spp., *Facklamia*

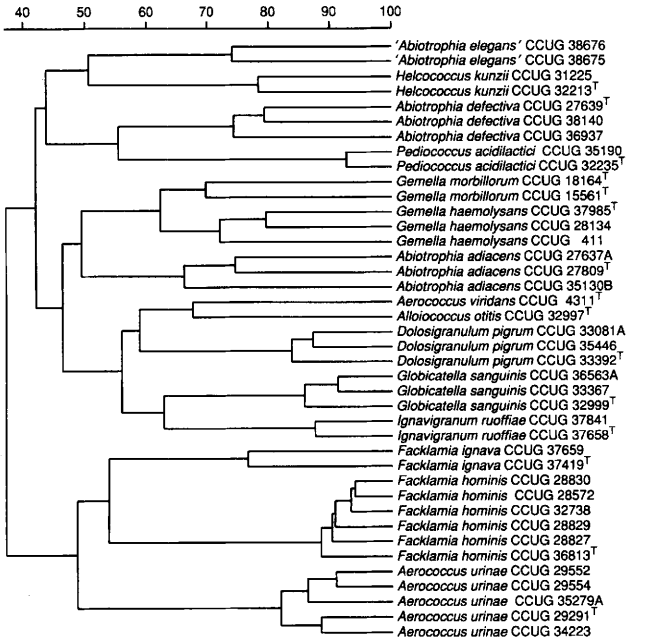

Fig. 1. Similarity dendogram based on whole-cell protein pattern of *Ignavigranum ruoffiae* sp. nov. and related species. Levels of correlation are expressed as percentages of similarity for convenience.

hominis and *Globicatella sanguinis* (Fig. 1). An examination of the cell wall of strain CCUG 37658^T revealed that the unknown coccus possessed a directly cross-linked murein based on L-lysine [type A1α, according to nomenclature of Schleifer & Kandler (1972)]. This murein structure is found in a number of Gram-positive catalase-negative cocci such as aerococci (Aguirre & Collins, 1992b), *Alloiococcus otitis* (Aguirre & Collins, 1992a), *Globicatella sanguinis* (Collins *et al.*, 1992) and *Abiotrophia defectiva* (Collins *et al.*, 1997). To assess the genealogical affinity between the two isolates and their relationship with other Gram-positive catalase-negative taxa, comparative 16S rRNA gene sequence analyses were performed. The almost complete gene sequences (> 1400 nucleotides) of the two clinical strains were determined and pairwise analysis revealed no base differences (i.e. 100% similarity) thereby showing their high phylogenetic relatedness. Sequence searches of GenBank and RDP databases revealed that the unknown bacterium was phylogenetically most closely associated with the lactic acid group of bacteria. A tree constructed by neighbour-joining depicting the phylogenetic affinity of the unknown coccus as exemplified by strain CCUG 37658^T is shown in Fig. 2. From the branching pattern of the tree the nearest relative of the unknown coccus was *Facklamia hominis*. The clustering together of these organisms occurred in 84% of 500 tree replications. *Globicatella sanguinis* formed a somewhat deeper subline branching off from the unknown coccus/*Facklamia hominis* line (supported by 83% bootstrap value). The next nearest relative of the

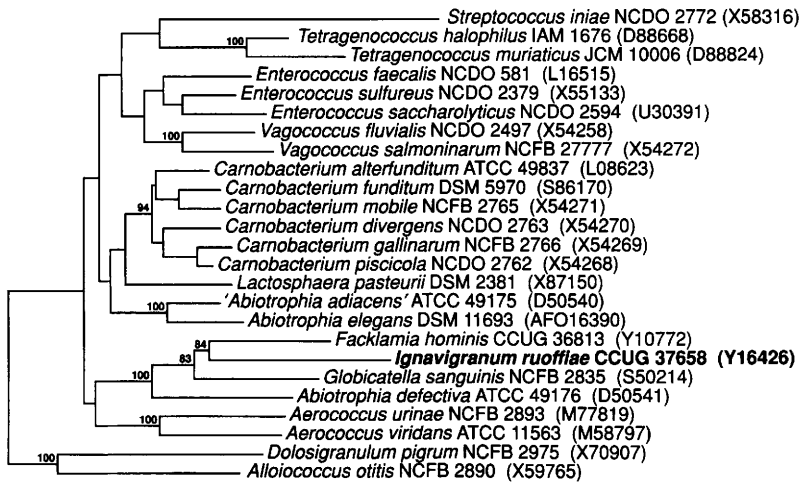

Fig. 2. Unrooted tree showing the phylogenetic relationships of *Ignavigranum ruoffiae* sp. nov. and some other low G+C content Gram-positive bacteria (all strains are type strains). The tree, constructed using the neighbour-joining method, was based on a comparison of about 1320 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points.

Table 1. Characteristics that differentiate *Ignavigranum ruoffiae* sp. nov. from *Facklamia hominis* and *Globicatella sanguinis*

Character	*I. ruoffiae*	*F. hominis*	*G. sanguinis*
Production of acid from:			
Glycogen	−	−	+
Lactose	−	−	v
Mannitol	v	−	+
Melibiose	−	−	+
Methyl-β-D-glucopyranoside	−	−	v
Pullulan	−	−	v
Raffinose	−	−	+
Ribose	−	−	+
Sorbitol	−	−	v
Sucrose	v	−	+
Trehalose	−	−	+
Hydrolysis of:			
Hippurate	−	+	+
Production of:			
Alanyl-phenylalanine-proline arylamidase	−	+	+
Arginine dihydrolase	+	+	−
α-Galactosidase	−	+	+
β-Galactosidase	−	+	+
Murein type:	A1α	A4α	A1α

aforementioned taxa corresponded to *Abiotrophia defectiva* (Fig. 2). Parsimony analysis was also performed and the grouping of the unknown coccus with *Facklamia hominis* and *Globicatella sanguinis* (as shown in Fig. 2) confirmed. All other significant associations shown in the neighbour-joining tree were also reproduced in the parsimony analysis (data not shown).

PAGE analysis of whole-cell proteins is an excellent tool for assessing close taxonomic relationships (e.g. species, subspecies). Using this approach it is clear that the two unknown isolates from human clinical sources clearly belong to a hitherto unrecognized Gram-positive catalase-negative species within the Lactic acid group of bacteria. By contrast, 16S rRNA permits an assessment of more distant phylogenetic affinities (e.g. interspecies relationships). From the 16S rRNA sequence analysis it is evident that the coccus has a close phylogenetic relationship with *Facklamia hominis*. The association between these taxa was supported by a bootstrap value of 84%. A sequence divergence value of 8·2% suggests this relationship is that of two phylogenetically closely related, but nevertheless separate, genera. Support for the separate generic status of the unknown bacterium comes from its possession of a Lys-directly cross-linked murein (type A1α). *Facklamia hominis* contains a cell wall

murein type L-Lys-D-Asp (type A4α) (Collins *et al.*, 1997). The unknown bacterium displayed a slightly higher 16S rRNA sequence similarity with *Globicatella sanguinis* (93·8%). A sequence divergence of > 6% and the branching pattern of the tree, however, indicates the relationship between these two taxa is that of two closely related, albeit different, genera. Additional evidence for the separateness of the unknown coccus from the genus *Globicatella* comes from its very characteristic phenotype, in particular its asaccharolytic nature. By contrast, *Globicatella sanguinis* is a highly saccharolytic organism capable of utilizing a broad range of carbohydrates (Collins *et al.*, 1992). It is pertinent to note that no other taxon examined possessed less than 8% 16S rRNA sequence divergence with the unknown bacterium. Examples of characteristics which serve to distinguish the unknown human bacterium from *Facklamia hominis* and *Globicatella sanguinis* are outlined in Table 1. Based on 16S rRNA sequence considerations and the distinctive phenotypic traits of the unknown coccus, we consider this bacterium merits classification as a new genus, for which the name *Ignavigranum ruoffiae* gen. nov., sp. nov. is proposed.

Description of *Ignavigranum* gen. nov.

Ignavigranum gen. nov. (Ig.na.vi.gra'num. L. adj. *ignavus* lazy, non-reacting; L. neut. n. *granum* grain, kernel; *Ignavigranum* lazy grain).

Cells are Gram-positive, non-spore-forming, non-motile, cocci occurring as single cells, in pairs or groups. Facultatively anaerobic and catalase-negative. Growth at 45 °C and in 6·5% NaCl. Weak acid production but no gas from glucose. Other carbohydrates generally not acidified. Hippurate, aesculin, gelatin and starch are not hydrolysed. Arginine dihydrolase, leucine arylamidase and urease are produced according to the API system. Voges–Proskauer negative. Nitrate is not reduced. The murein type is L-lysine-direct (A1α). The G+C content of DNA is 40 mol% (T_m). The type species of the genus is *Ignavigranum ruoffiae*. As determined by 16S rRNA gene sequence analysis, the genus *Ignavigranum* belongs to the Lactic acid group of bacteria with low DNA G+C contents, and is phylogenetically closely related to *Facklamia hominis* and *Globicatella sanguinis*, but may be distinguished from the latter two taxa using traits shown in Table 1.

Description of *Ignavigranum ruoffiae* sp. nov.

Ignavigranum ruoffiae (ru.off'iae. Named after Kathryn L. Ruoff an American microbiologist, in recognition of her contributions to the microbiology of Gram-positive cocci).

Cells are Gram-positive, ovoid in shape, occurring as single cells, in pairs or groups. Cells are non-spore-forming and non-motile. Some strains show satelliting enhancements. Facultatively anaerobic and catalase-negative. Strains grow at 45 °C and 6·5% NaCl. Acid produced weakly from glucose. Acid is not produced from L-arabinose, D-arabitol, cyclodextrin, glycogen, lactose, D-mannose, melibiose, melezitose, pullulan, D-raffinose, D-ribose, sorbitol, tagatose, trehalose or D-xylose. Some strains produce acid from mannitol and sucrose. Using commercial API systems arginine dihydrolase, leucine arylamidase, pyroglutamic acid arylamidase and urease activity is detected. Alanyl-phenylalanine-proline arylamidase, cystine arylamidase, chymotrypsin, α-fucosidase, alkaline phosphatase, α-galactosidase, β-galactosidase, β-galacturonidase, β-glucuronidase, glycyl-tryptophan arylamidase, lipase C14, β-mannosidase, trypsin and pyrazinamidase activity is not detected. Aesculin, hippurate, gelatin and starch are not hydrolysed. Arginine dihydrolase and urease are negative in conventional tests. Voges–Proskauer negative. Nitrate is not reduced. The G+C content of DNA is 40 mol%. The cell-wall murein type is L-lysine-direct (A1α). Isolated from clinical sources. Habitat not known. The type strain is CCUG 37658^T. Strain CCUG 37658^T was isolated from a wound infection.

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References

Aguirre, M. & Collins, M. D. (1992a). Phylogenetic analysis of an unknown bacterium from human middle ear fluid: description of *Alloiococcus otitis* gen. nov., sp. nov. *Int J Syst Bacteriol* **42**, 79–83.

Aguirre, M. & Collins, M. D. (1992b). Phylogenetic analysis of some *Aerococcus*-like organisms from urinary tract infections: description of *Aerococcus urinae* sp. nov. *J Gen Microbiol* **138**, 401–405.

Aguirre, M., Morrison, D., Cookson, B. D., Gay, F. W. & Collins, M. D. (1993). Phenotypic and phylogenetic characterization of some *Gemella*-like organisms from human infections: description of *Dolosigranulum pigrum* gen. nov., sp. nov. *J Appl Bacteriol* **75**, 608–612.

Collins, M. D., Aguirre, M., Facklam, R. R., Shallcross, J. & Williams, A. M. (1992). *Globicatella sanguis* gen. nov., sp. nov., a new gram-positive catalase-negative bacterium from human sources. *J Appl Bacteriol* **73**, 422–437.

Collins, M. D., Facklam, R. R., Rodrigues, U. M. & Ruoff, K. L. (1993). Phylogenetic analysis of some *Aerococcus*-like organisms from clinical sources: description of *Helcococcus kunzii* gen. nov., sp. nov. *Int J Syst Bacteriol* **43**, 425–429.

Collins, M. D., Falsen, E., Lemozy, J., Åkervall, E., Sjödén, B. & Lawson, P. A. (1997). Phenotypic and phylogenetic characterization of some *Globicatella*-like organisms from human sources: description of *Facklamia hominis* gen. nov., sp. nov. *Int J Syst Bacteriol* **47**, 880–882.

Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.

Facklam, R. R. & Elliot, J. A. (1995). Identification, classification,

and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin Microbiol Rev* **8**, 470–495.

Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.

Garvie, E. I. (1978). *Streptococcus raffinolactis* (Orla-Jensen and Hansen); a group N streptococcus found in raw milk. *Int J Syst Bacteriol* **28**, 190–193.

Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Modern Microbial Methods. Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.

Roggenkamp, A., Abele-Horne, M., Trebesius, K. H., Tretter, U., Autenreith, I. B. & Belesemann, J. (1998). *Abiotrophia elegans* sp. nov., a possible pathogen in patients with culture-negative endocarditis. *J Clin Microbiol* **36**, 100–104.

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cells walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.