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Anaerococcus nagyae sp. nov., isolated from human clinical specimens

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ABSTRACT

We describe a new *Anaerococcus* species isolated from human clinical specimens. Analyses of 16S rRNA gene sequences of three strains showed <98% similarity with its closest relative *Anaerococcus octavius*. Phylogenetically the isolated strains form a cluster and can be differentiated from other species of the genus *Anaerococcus* based on its phenotypic characteristics and its MALDI-TOF MS profile. We propose the name *Anaerococcus nagyae*, with *A. nagyae* DSM101193 (accession number KU043522) as the type strain.

1. Introduction

Gram-positive anaerobic cocci (GPAC) are part of the commensal human microbiota and play a role in human infectious diseases [1,2]. From all anaerobic bacteria isolated from human clinical specimens, about 30% are GPAC [2]. The genus *Anaerococcus* is one of the genera into which six species, which first belonged to the genus *Peptostreptococcus*, were assigned to [3]. Since that time several new species belonging to the genus *Anaerococcus* have been described. Two new species isolated from human clinical specimens were described based on their 16S rRNA sequence and phenotypic features; *Anaerococcus murdochii* [4] and *Anaerococcus degenerii* [5]. Another species isolated from human clinical specimens was described using whole genome sequencing: *Anaerococcus provenciensis* [6]. Two new species were isolated from human faecal samples; *Anaerococcus pacaensis* [7] and *Anaerococcus senegalensis* [8]. At this moment the genus consists out of 11 species. Recently, Johnson et al. [9] determined that several genera, including the genus *Anaerococcus*, of GPAC form one group sharing the same phylogenetic, biochemical and chemotaxonomic features. To accommodate this group of genera the family of *Peptoniphilaceae* was proposed.

Furthermore, the nucleotide database of the National Center of Biotechnology (NCBI) contains several 16S rRNA gene sequences of *Anaerococcus* sp. that are not described. One of these sequences was submitted by La Scola et al. [10], strain *Anaerococcus* sp. 8405254 (accession number HM587319), which was isolated from a human clinical specimen. Another one was submitted by Fuji et al. [11], strain *Anaerococcus* sp. SJ-2013 (accession number AB853090). Sequence analyses showed that these two strains belong to the same species. The latter was isolated from the axila and releases a large amount of 3-hydroxy-3-methyl-hexanoic-acid (HMHA). Within the European Network for the Rapid Identification of Anaerobes project (ENRIA) we encountered three strains which are similar to these two *Anaerococcus* strains. In this study we describe the biochemical features of these strains, their phylogenetic position and their main spectral profile (MSP) which can be used to identify this bacterium by Matrix Assisted Laser Desorption Ionization time-of-flight Mass Spectrometry (MALDI-TOF MS). For this new species we propose the name *Anaerococcus nagyae*.

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2. Material and methods

2.1. Bacterial strains and growth conditions

Two of the studied strains originate from the Centre Hospitalier Universitaire de Montpellier, France (FR3773 and FR4046), and one from the University Medical Center Groningen, The Netherlands (UMCG-3741). Within the ENRIA project each strain was assigned an ENRIA number, ENR0652, ENR0669 and EN0686, respectively. Strain ENR0652 was isolated from a 28 years old woman, with a fungal infection of the groin with secondary bacterial infection. ENR0669 was isolated from a 51 years old man with a post nephrourecterectomy wound infection after carcinoma. In both cases the infection involved a mixed aerobic/anaerobic infection. Species isolated together with strain ENR0652 were *Propionibacterium avidum*, *Klebsiella pneumoniae* and *Corynebacterium tuberculostearicum*. Strain ENR0669 was simultaneously isolated with *Propionibacterium acnes* and *Staphylococcus lugdunensis*. Strain ENR0686 was isolated from a positive blood culture from a 84 years old women suffering from influenza A and ischemia in conjunction with *P. acnes*.

Strains were cultured on Brucellla Blood Agar (BBA, Mediaproducts, Groningen, The Netherlands) and incubated under anaerobic conditions (80% N_2, 10% H_2 and 10% CO_2) for 48 h at 37 °C. Strains were stored in Microbank™ vials (Pro-Lab Diagnostics, Cheshire, United Kingdom) at- 80 °C until use.

2.2. Biochemical features

The special-potency disk pattern of the strains, used for preliminary identification of anaerobes, for the antibiotic disks kanamycin (1000 mg), vancomycin (5 mg) and colistin (10 mg) was determined according to the Wadsworth manual [12]. Biochemical features of the strains were determined using the Rapid ID 32A (BioMerieux, France) and the ANC-card (BioMerieux, France). The latter was included to study carbohydrate fermentation. Both test systems were used according to the manufacturers instructions.

2.3. MALDI-TOF MS

A full extraction of the bacterial strains was performed as described previously [13]. Briefly, a bacterial suspension was prepared by suspending an 1 μl loopfull of bacteria in 300 μl sterile distilled water. After obtaining a homogeneous suspension, 900 μl absolute ethanol was added. The suspension was centrifuged at 13,000 g for 2 min and the supernatant was discarded. The centrifugation step was repeated and the remaining supernatant was carefully removed by pipetting. The pellet was resuspended in 30 μl 70% formic acid and an equal amount of acetonitrile. The obtained suspension was centrifuged at 13,000 g for 2 min. 1 μl of the supernatant was spotted twelve times on a stain-less steel target and was left to dry at ambient temperature. Immediately after drying 1 μl matrix HCCA (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoro-acetic acid) was added to the spots and left to dry at ambient temperature. A total of 36 spectra were obtained using the microflex MALDI-TOF MS system (Bruker Daltonik, GmbH, Bremen, Germany). Prior to the measurements the system was calibrated using a bacterial test standard (BTS, Bruker Daltonik). The obtained spectra were analyzed using flexanalysis 3.3.80.0. For each spectrum the appropriate method was chosen (MBT-standard) and smoothing and baseline subtraction were performed. Spectra were manually checked for flat liners and outliers. The peak shift between the different spectra was allowed to be no more than 500 ppm. Spectra of questionable quality were removed. From the remaining spectra (which should be at least 20) a main spectral profile (MSP) was calculated in Biotyper 3.0. A dendrogram of the obtained MSPs was calculated including MSPs of other *Anaerococcus* species. The obtained MSPs were compared with MSPs already present in the database. Log scores were interpreted as recommended by the manufacturer; log score $\geq$2 as reliable species identification, log score between 1.7 and 2.0 as reliable genus identification and log scores $\leq$1.7 as no reliable identification.

2.4. 16S rRNA gene sequencing and phylogenetic analyses

DNA of the strains was isolated as described previously [14]. The 16S rRNA gene of the three strains was amplified using universal primers described by Schuurman et al. [15]. The obtained sequences were aligned against sequences of reference strains derived from the Nucleotide Center of Biotechnology (NCBI) and the Ribosomal Database Project (RDP) [16] using the ARB-software [17]. A phylogenetic tree was constructed using the neighbour joining method with Jukes Cantor correction, including bases between *Escherichia coli* positions 211 and 1353. The topology of the tree was calculated using bootstrap analyses of 1000 replicate trees.

3. Results

3.1. Biochemical features

After anaerobic incubation, the strains showed a divers colony morphology, yellowish or white/grey, with a diameter of 2–4 mm. Cells stained gram-positive, were often arranged in pairs and had a coccoid morphology with a diameter of >0.6 μm. Strains were sensitive for kanamycin (1000 mg) and vancomycin (5 mg), but resistant to colistin (10 mg). The results of the biochemical tests are presented in Table 1. A positive reaction for indole and pyroglutamic acid arylamidase was observed. Strains showed negative reactions for catalase, urease, alkaline phosphatase, arginine dihydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucuronidase, proline arylamidase, tyrosine arylamidase, glutamyl glutamic acid, serine arylamidase, arginine arylamidase, leucine arylamidase and histidine arylamidase. All strains were able to ferment mannose, but not raffinose and glucose.

The key reactions to differentiate *A. nagyae* from other *Anaerococcus* species are presented in Table 2. A positive indole reaction differentiates *A. nagyae* from the majority of the *Anaerococcus* species. A negative reaction for arginine arylamidase, leucine arylamidase and histidine arylamidase differentiates *A. nagyae* and *Anaerococcus hydrogenalis* from *Anaerococcus vaginalis* and *A. senegalensis*. *A. nagyae* can be differentiated from *A. hydrogenalis* by a positive reaction of pyroglutamic acid arylamidase and its inability to ferment raffinose.

3.2. Phylogenetic analyses

A search in NCBI using Blastn yields as closest official described species *Anaerococcus octavius* NCTC9810. The sequence similarities of the strains ENR0652, ENR0669 and ENR0686 with *A. octavius* were 97,3%, 96,3% and 96,2%, respectively. The sequence similarity between *Anaerococcus* sp. 840254 (acc. nr. HM587319) and the clinical isolates ENR0652, ENR0669 and ENR0686, is 99,9%, 100% and 100%, respectively. Furthermore, a search showed that the sequences of *Anaerococcus* sp. 840254 and *Anaerococcus* SJ-2013 (acc. nr. AB853090) are identical to each other. The phylogenetic position of our three clinical isolates, the two *Anaerococcus* strains similar to our strains and other A*naerococcus* species is shown in Fig. 1.

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Table 1
The biochemical features of *Anaerococcus nagyae* and other *Anaerococcus* species.

	IND	CAT	URE	ALP	ADH	α-Gal	β-Gal	α-Glu	β-Gur	ArgA	ProA	LeuA	PyrA	TyrA	HisA	GGA	SerA	glu	man	raff
Anaerococcus nagyae	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−
Anaerococcus degenerii[a]	−	−	−	+	-w	−	−	−	−	+	−	+	−	−	+	−	−	+	+	−
Anaerococcus murdochii[b]	−	−	−	+	+	−	+	−	−	+	−	+	+	−	+	−	v	+	+	−
Anaerococcus tetradius[c]	−	nd[g]	+	−	−	+	−	+	+	+	−	−	+	w	+	nd	nd	−	+	−
Anaerococcus lactolyticus[c]	−	−	+	−	−	−	+	−	−	+	−	−	−	−	−	−	v	+	+	−
Anaerococcus octavius[c]	−	v	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	+	−
Anaerococcus prevotii[c]	−	+	v	−	−	+	−	+	+	+	−	−	+	w	+	−	+/w	−	+	+
Anaerococcus vaginalis[c]	v	w	−	-w	+	−	−	−	−	+	−	+	−	−	+	−	v	+	−	−
Anaerococcus hydrogenalis[c]	+	−	v	-w	−	−	−	v	−	−	−	−	−	−	−	−	−	+	+	+
Anaerococcus pacaensis[d]	−	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Anaerococcus senegalensis[e]	+	+	+	+	+	−	−	−	−	+	−	+	w	−	+	−	−	nd	+	−
Anaerococcus provenciensis[f]	−	nd	−	+	+	−	+	−	+	+	−	+	+	−	+	−	−	nd	+	−

IND, indole; CAT, catalase; URE, urease; ALP, alkaline phosphatase; ADH, arginine di-hydrolase; α-gal, α-galactosidase; β-gal, β-galactosidase; α-glu, α-glucosidase; β-gur, β-glucuronidase; ArgA, arginine arylamidase; ProA, proline arylamidase; LeuA, leucine arylamidase; PyrA, pyroglutamic acid arylamidase; TyrA, tyrosine arylamidase; HisA, histidine arylamidase; GGA, glutamyl glutamic acid; SerA, serine arylamidase; glu, glucose; man, mannose; raff, raffinose.

[a] Veloo et al. (Anaerobe 2015; 33:71–75).
[b] Song et al. (J Clin Microbiol 2007; 45:1746–1752).
[c] Wadsworth-KTL Anaerobic Bacteriology Manual, Jousimes-Somer et al. (6th edition, 2002).
[d] Pagnier et al. (Stand Genomic Sci 2013; 8:548–560).
[e] Lagier et al. (Stand Genomic Sci 2012; 6:116–125).
[f] Pagnier et al. (Stand Genomic Sci 2014; 9:1198–1210).
[g] nd, not determined.

Table 2
Key biochemical features to differentiate *Anaerococcus nagyae* from other indole-producing *Anaerococcus* species.

	IND	CAT	URE	ALP	ADH	ArgA	LeuA	PyrA	HisA	SerA	glu	man	raff
Anaerococcus nagyae	+	−	−	−	−	−	−	+	−	−	−	+	−
Anaerococcus vaginalis[a]	v	w	−	-w	+	+	+	−	+	v	+	−	−
Anaerococcus hydrogenalis[a]	+	−	v	-w	−	−	−	−	−	−	+	+	+
Anaerococcus senegalensis[b]	+	+	+	+	+	+	+	w	+	−	nd[c]	+	−

IND, indole; CAT, catalase; URE, urease; ALP, alkaline phosphatase; ADH, arginine di-hydrolase; ArgA, arginine arylamidase; LeuA, leucine arylamidase; PyrA, pyroglutamic acid arylamidase; HisA, histidine arylamidase; SerA, serine arylamidase; glu, glucose; man, mannose; raff, raffinose.

[a] Wadsworth-KTL Anaerobic Bacteriology Manual, Jousimes-Somer et al. (6th edition, 2002).
[b] Lagier et al. (Stand Genomic Sci 2012; 6:116–125).
[c] nd, not determined.

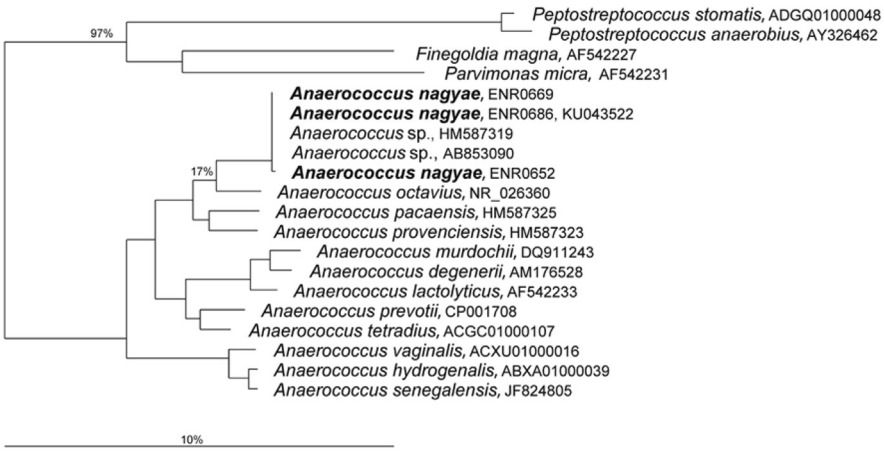

Fig. 1. Phylogenetic tree showing the relation between the different *Anaerococcus* species. Strains belonging to the new species *Anaerococcus nagyae* are shown in bold. The tree was constructed using the neighbor joining method and alignments between *Escherichia coli* positions 211–1353. Only bootstrap values of >90% are shown. The bar indicates 10% sequence divergence.

3.3. MALDI-TOF MS

The dendrogram calculated from the obtained MSPs (Fig. 2) shows that the three *A. nagyae* strains form a separate cluster within the genus *Anaerococcus*. The log scores between the three strains were >2.3 and with other *Anaerococcus* species present in the database <1.7.

4. Discussion

The three strains described in this study have a sequence similarity <98% with their closest relative *A. octavius* and form a homogenous phylogenetic cluster (Fig. 1) with sequence similarities with each other between 99 and 100%. The two previous described strains by La Scola et al. [10] and Fuji et al. [11] are within the same

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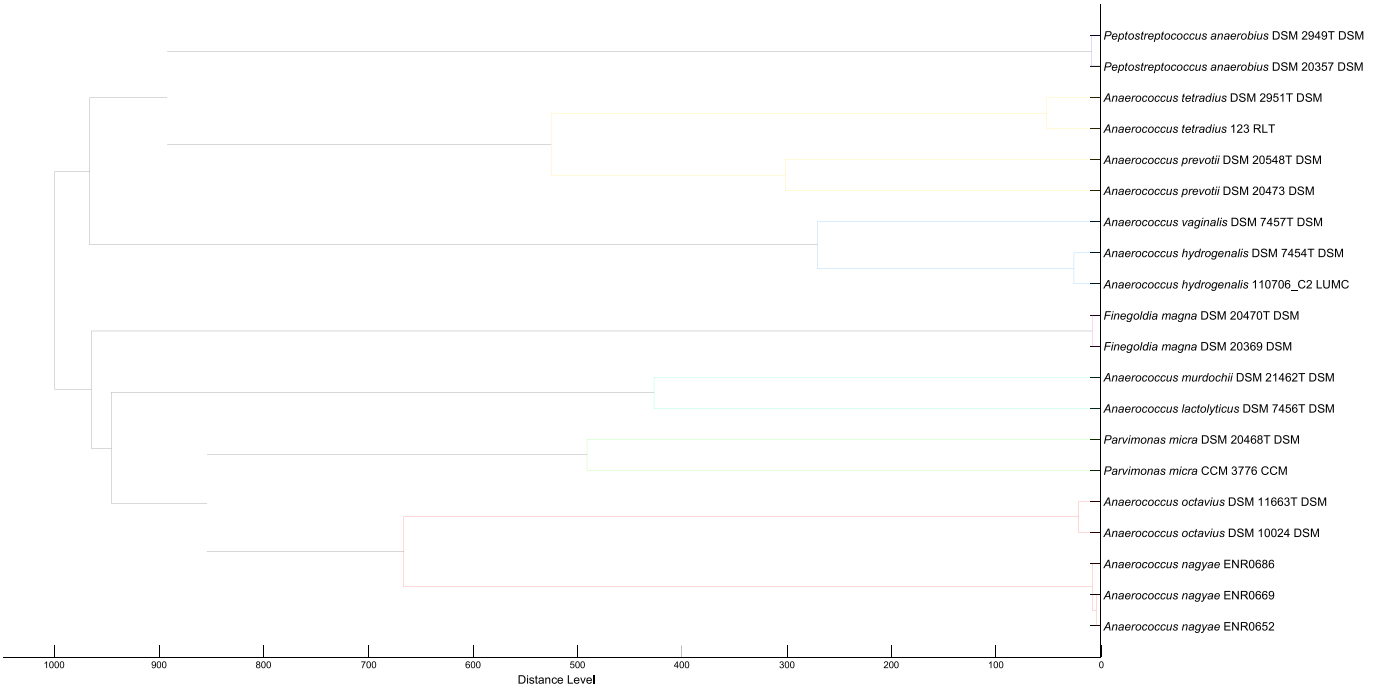

Fig. 2. Dendrogram of the MSPs of the three *Anaerococcus nagyae* strains and related *Anaerococcus* species.

phylogenetic cluster. Stackebrandt and Ebers [18] described that the sequence cut of value for defining a new species is about 98.7%. Furthermore, the three clinical isolates described in this study also form a phenotypically homogeneous cluster and can be differentiated from other *Anaerococcus* species based on their biochemical features. Rosselló-Mora and Amann [19] defined a phylo-phenetic species concept: '*a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property*'. Our strains meet the conditions set by Stackebrandt and Ebers [18] and Rosselló-Mora and Amann [19], in order to be named a new species. Furthermore, they can be easily identified and differentiated from other *Anaerococcus* species using MALDI-TOF MS.

Description of *Anaerococcus nagyae* (named after Elisabeth Nagy for her contribution to the anaerobic bacteriology). After five days of incubation on BBA in an anaerobic environment colonies are diverse, yellowish or white/grey, smooth and 2–4 mm in diameter. The cells stain gram-positive and the morphology observed in the gram-stain are cocci with a diameter >0.6 μm. Cells are mostly arranged in pairs. All strains are sensitive for kanamycin (1000 mg) and vancomycin (5 mg), but resistant to colistin (10 mg). A positive reaction was observed for indole and pyroglutamic acid arylamidase. A negative reaction for catalase, urease, alkaline phosphatase, arginine di-hydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucuronidase, proline arylamidase, tyrosine arylamidase, glutamyl glutamic acid, serine arylamidase, arginine arylamidase, leucine arylamidase and histidine arylamidase. All strains were able to ferment mannose, but not raffinose and glucose. The type strain is DSM101193 = ENR0686 = UMCG-3741 (accession number KU043522).

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