ORIGINAL ARTICLE

Whole cell protein and partial 16S rRNA gene sequence analysis suggest the existence of a second *Rothia* species

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Objective: To subject ten clinical isolates grouped together based on their biochemical and microbiological profile to further investigations aimed at correct species identification.

Methods: The 16S rRNA gene was partially sequenced using nested amplification. Whole cell protein analysis (SDS-PAGE) and cluster analysis were performed on the 10 strains and also for comparison on 31 reference strains. The API Coryne biochemical kit as well as API 20 Strep were used for analysis of the phenotypic diversity of the strains by use of computerized numerical identification procedures. Antibiotic susceptibility testing was performed using a standardized disk diffusion test.

Results: The 265–556-bp-long 16S rRNA gene sequences of all 10 strains showed highest similarity to *Rothia dentocariosa*. Three strains showed complete identity between the sequences obtained and the sequence of the type strain of *Rothia dentocariosa* 16S rRNA gene (M59055), and the other seven ranged between 99.7% and 98.3% similarity. Detailed analysis of the sequences revealed a clustering of the strains into two groups. One group consisted of four isolates with the highest degrees of similarity with the reference strain (type I), while the members of another group (type II) showed differences in their nucleotide sequence at four distinct positions in the variable V7 region. T was replaced by C at position 597, C by T at position 608, T by C at position 610, and G by A at position 684 (position numbers according to reference sequence M59055, EMBL/GenBank). Whole cell protein analysis (SDS-PAGE) and cluster analysis also segregated the 10 *Rothia dentocariosa* strains into two different clusters, with one cluster containing all four strains belonging to 16S rRNA gene type I, and a second cluster containing all six strains belonging to 16S rRNA gene type II.

Conclusions: Partial sequence data of the 16S rRNA gene as well as whole cell protein analysis showed a subdivision of the *Rothia* species into two groups, genomovar I (*Rothia dentocariosa sensu stricto*) and genomovar II, a possible new *Rothia* species.

Key words: *Rothia dentocariosa*, 16S rRNA gene sequence, whole cell protein pattern

INTRODUCTION

Rothia dentocariosa is an opportunistic pathogen which has been found to cause septicemia and endocarditis [1–9] as well as other infections [10–14]. This species is a normal inhabitant of the oral cavity and the pharynx. In the present study we have identified 10 strains submitted to a reference laboratory for speciation as *R. dentocariosa*. Three of the patients yielding *R. dentocariosa* isolates were diagnosed as cases of endocarditis.

Many bacterial isolates from immunocompromised patients represent opportunistic microorganisms which may be difficult to speciate using routine procedures [15–19]. At the same time, many of these infections constitute life-threatening conditions which must be treated adequately. *R. dentocariosa* represents one such

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microorganism which sometimes might be overlooked by the microbiology laboratory. It is not even included in the species list for API Coryne [17]. We have therefore reviewed the possibilities for species identification of *R. dentocariosa* in the clinical bacteriology laboratory and offer a computer program for numerical identification, IDBact (Web page: "http//www.ki.se/labmed/clim/main.htm", see instructions under "Get a free copy . . ."), using the results of API Coryne. We have also found evidence for a subdivision of the species *R. dentocariosa* into two homogeneous groups by both 16S rRNA gene sequencing and whole cell protein analysis.

MATERIALS AND METHODS

Bacterial strains

The Culture Collection, University of Gothenburg (CCUG), Sweden, accepts isolates from clinical laboratories in Scandinavia and elsewhere for identification and characterization. Biochemical and microbiological profiles of the strains yield a direct species identification or a categorization to a tentative EF group (EF, Enevold Falsen, curator of the collection). From one such tentative group, EF-41, 10 strains of pleomorphic Gram-positive rods obtained from various clinical specimens were included in the present study. The strains were sent to CCUG for speciation from clinical microbiology laboratories in Sweden over a period of several years. Since the collection included strains sent from the present laboratory (Karolinska Institute/Hospital), we chose this tentative group for a more detailed collaborative analysis including 16S rRNA gene sequencing.

The 10 strains are listed in Table 1, with their CCUG identification number, age and sex of the patient, type of specimen yielding the culture isolate, and information about relevant diseases. It was not possible to trace treatment regimens satisfactorily.

When the results of the 16S rRNA gene sequencing as described in this paper provided a direct species identification, we included the reference strain of *R. dentocariosa*, ATCC 17931 (NCTC 10917, CCUG No. 35437), in the study. This strain originates from a human caries lesion and was originally isolated by Geneviève D. Roth. The 16S rRNA gene of the strain was sequenced in 1990 by D. Yang and C.R. Woese, and the 1476-bp-long sequence is available in the EMBL/GenBank databases under accession number M59055.

Twenty-eight strains of representative *Arcanobacterium*, *Actinomyces*, and *Stomatococcus mucilaginosus* from the CCUG collection, as well as three additional *R. dentocariosa* strains, were included in whole cell protein analysis (SDS-PAGE) (Figure 1). The strains were selected because of their close phenetic and phylogenetic relationship with *R. dentocariosa* as determined in preliminary experiments using a wide range of bacterial strains from CCUG representing different species of Gram-positive bacteria.

Microbiological culture media

The CCUG group 41 strains were cultured on McLeod (hemin plates) or on 5% horse blood Columbia agar in a CO_2 atmosphere and on blood agar plates anaerobically for 24–48 h. *Stomatococcus mucilaginosus* was grown for 24 h in a CO_2-containing atmosphere on 5% horse blood Columbia agar. *Actinomyces* (except *Actonomyces israelii*) strains were grown for 24–48 h in CO_2 on 5% horse blood Columbia agar. *Actinomyces israelii* was cultured anaerobically for 48–36 h on 5% horse blood Columbia agar. Aliquots of subcultures were suspended in a special freeze broth contained in small plastic tubes and stored at −80°C.

For antibiotic susceptibility testing using the disk diffusion method (standardization according to SRGA [20,21]), strains were inoculated onto Isosensitest agar (Oxoid AB, Sjöängsvägen 7, 192 72 Sollentuna,

Table 1 Clinical isolates of Gram-positive pleomorphic strains included in the study

No.	CCUG	Age	Sex	Specimen	Disease
1	30072	41	M	Blood	Alcohol abuse, brain contusion, fever, epiglottitis
2	33996	NI[a]	NI[a]	Bucca	NI[a]
3	32449	42	F	Pharynx	Heart transplantation
4	28595	30	M	Blood, valve	Intravenous drug abuse, prosthetic mitral valve, endocarditis, died
5	30754	59	M	Blood	Heart transplant, aortic valve failure, endocarditis with peripheral embolism
6	27465	63	M	Blood	Chronic glomerulonephritis, uremia, septic peripheral embolism
7	33543	50	F	Blood	Adrenal cortical cancer, immunosuppressed
8	17835	NI[a]	NI[a]	CSF[b]	NI[a]
9	35343	43	M	Mitral valve	Mitral valve prolapse, biopsy-verified endocarditis
10	25688	35	F	Peritoneal dialysis	Diabetes, nephropathy, peritoneal dialysis, then hemodialysis, fever

[a] NI, no further information available. [b] Cerebrospinal fluid.

Sweden) containing 5% horse blood, and the antibiotic disks applied to the surface. After preincubation for 30 min at room temperature, the Petri dishes were incubated at 37 °C overnight.

Biochemical test profile

The identification kits API 20 Strep and API Coryne (BioMérieux SA, Lyon, France, purchased from Triolab AB, Box 2109, S-431 02 Mölndal, Sweden) were tested

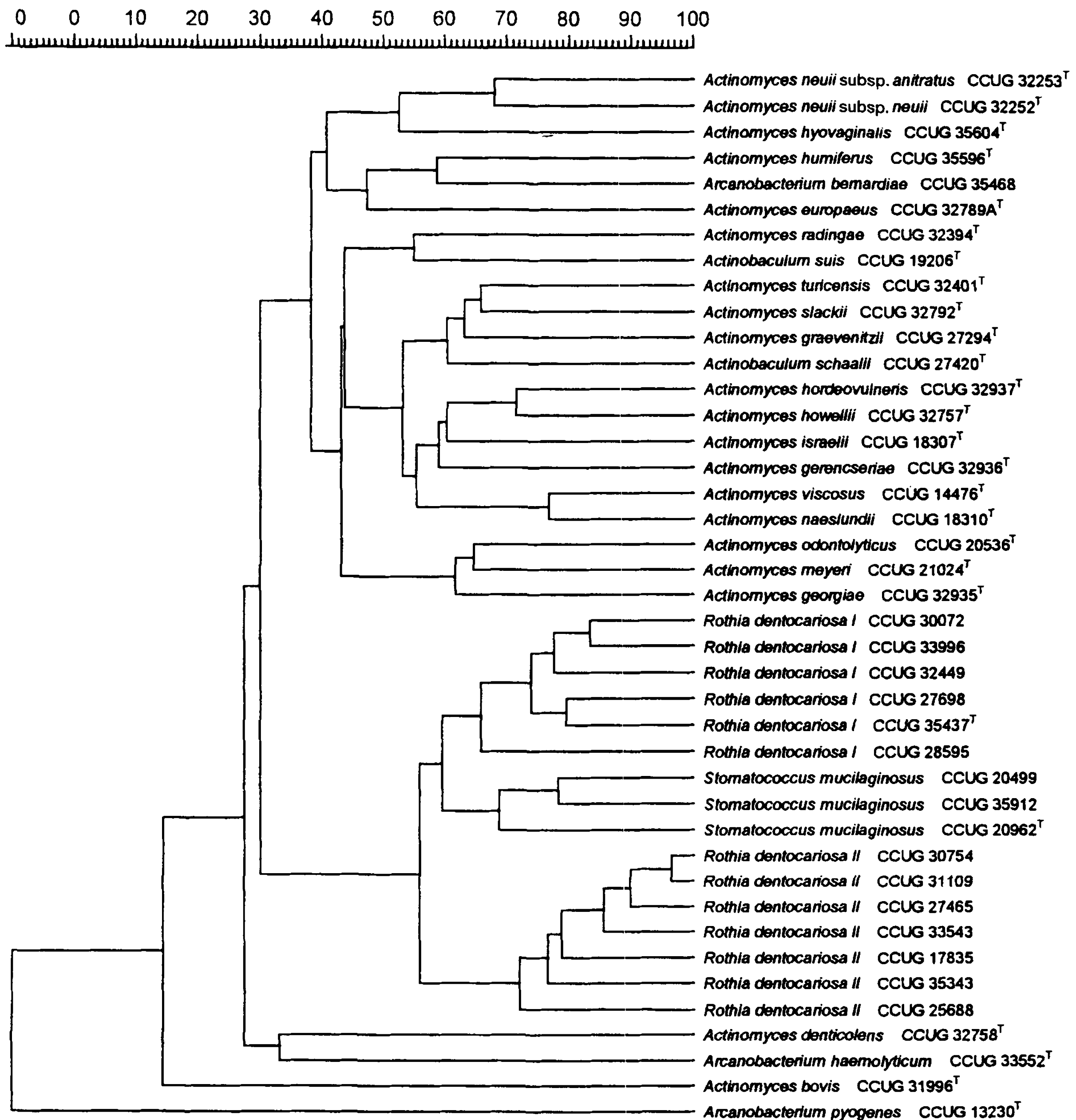

Figure 1 Dendrogram derived from unweighted pair-group average linkage of correlation coefficients (expressed for convenience as percentages) for whole cell protein patterns of the strains examined. The nucleotide sequence data reported in this paper for genotype II of *Rothia dentocariosa* as represented by strain CCUG 25688 will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y12925.

regarding their suitability for testing the 10 Gram-positive bacterial strains. Neither of these two identification schemes included *R. dentocariosa*. API Coryne is primarily designed for Gram-positive rods.

Antibiotic susceptibility testing

The antibiotic susceptibilities of the 10 strains were determined using the disk diffusion method according to Swedish standardization (Swedish Reference Group for Antibiotics, SRG, Swedish Medical Association [21]) with interpretation adjusted for species groups [21–25]. The strains were inoculated onto Isosensitest agar containing 5% horse blood. Antibiotic disks were placed on the surface, followed by preincubation at room temperature for 30 min and then by overnight incubation at 37°C. Inhibition zone diameter values were read by a pair of calipers in millimeters with one decimal. Interpretation of susceptibility followed zone diameter breakpoints given for coryneform bacteria according to SRGA.

Antibiotic disks were purchased from Oxoid Ltd (Oxoid AB, 192 72 Sollentuna, Sweden). The following antibiotics were included: penicillin G (disk content 10 μg), penicillin V (10 μg), ampicillin (10 μg), piperacillin (30 μg), cefuroxime (30 μg), cefotaxime (30 μg), imipenem (10 μg), gentamicin (30 μg), tetracycline (30 μg), erythromycin (15 μg), clindamycin (15 μg), chloramphenicol (30 μg), rifampicin (5 μg), vancomycin (30 μg), and ciprofloxacin (10 μg).

Whole cell protein analysis (SDS-PAGE)

Organisms were grown under optimal conditions and harvested as early stationary-phase cultures. Time, atmosphere and medium for culture are specified above. The preparation of cellular protein extracts (ultrasound treatment of all Gram-positive organisms), polyacrylamide gel electrophoresis (PAGE), densitometric analysis, normalization and interpolation of the protein profiles, and a numerical analysis, were performed as described by Pot et al [26] by using the Hoefer SE 600 vertical slab gel unit, Pharmacia-LKB UltroScan XL (Pharmacia-LKB, Uppsala, Sweden) and the GelCompar 3.0 software package (Applied Maths, Kortrijk, Belgium). The levels of similarity between pairs of traces were expressed by the Pearson product moment correlation coefficient converted for convenience to a percentage value.

DNA preparation from bacterial strains

Bacterial strains were cultured aerobically and anaerobically on blood agar base for 2 days at 37°C. The bacteria were harvested and suspended in a special deep freeze broth (Nutrient Broth No. 2, Oxoid CM67, 85%, and glycerol, 15%, v/v) and frozen at −70°C. Aliquots (25 μL) of the bacterial suspension were diluted with an equal volume of distilled water and then boiled for 10 min in a heating block at 100°C. Five microliters of these lysed bacterial cells was used as the template in the first PCR amplification.

16S rRNA gene sequencing

Using the Perkin-Elmer GeneAmp PCR system 2400, a nested PCR amplification was performed consisting of 2 × 30 cycles with denaturation at 96°C for 15 s (for 2 min in each first cycle) with annealing at 60°C when using the first pair of primers (U1–U8, Table 2) and at 65°C when using the second pair of primers (U2–U8, Table 2) for 30 s and with extension at 72°C for 1.5 min (for 5 min in each last cycle).

The reaction mixture (50 μL) contained Tris 10 mM pH 8.5, KCl 50 mM, $MgCl_2$ 2 mM, Tween-20 0.1%, 10 pmol of each outer primer (U1–U8) or 5 pmol of each internal primer (U2–U8) and 1.5 U *Taq* polymerase. One microliter of amplified material from the first set of 30 cycles was used as template in the second amplification step. Five microliters of the amplified product from the second step was controlled for presence of DNA by running on a 1% agarose gel containing ethidium bromide and then viewing under UV light.

Primer pairs for the nested PCR amplification were as follows. For the primary amplification between universal regions U1 and U8, the forward U1 primer 593 II [27] was used together with the biotinylated

Table 2 Primers used for PCR amplification and partial sequencing of the 16S rRNA gene

Primer	16S region	Nucleotide sequence[a]	Application
593 II	U1	5′-GTTTGATCCTGGCTCAGGAYDAACG-3′	PCR
611	U2	5′-USP[b]-CCRIACTYCTACGGRRGGCAGC-3′	PCR
621 b	U8	5′-Biotin-RSP[b]-SSTACGGITACCTTGTTACGAC-3′	PCR
USP[b]		5′-FITC-CGTTGTAAAACGACGGCCAG-3′	Sequencing
RSP[b]		5′-FITC-CACAGGAAACAGCTATGACC-3′	Sequencing

[a] Y=C/T, D=A/G/T, R=A/G, S=C/G, FITC=fluorescein isothiocyanate.
[b] USP and RSP are the nucleotide sequences of the universal and reverse sequencing handles, which are one and four nucleotides shorter, respectively, than the commercially available sequencing primer USP and reverse sequencing primer RSP.

reversed U8 primer 621b [28]. In the second round, the amplification was performed between the universal regions U2 and U8 using the forward U2 primer 611 and the same reversed U8 primer as above, 621b (Table 2).

The biotinylated PCR products (40 μL) were bound to streptavidin-coated magnetic beads (Dynabeads M280-Streptavidin, Dynal AS, Norway). Single-stranded DNA for sequencing was obtained following the procedures given by the manufacturer (Dynal AS).

The sequencing reaction was performed in a microtiter plate on a water-bath using the AutoRead Sequencing Kit from Pharmacia Biotech (Sollentuna, Sweden) according to the instructions of the manufacturer. This kit utilizes the M13 universal primer, which is fluorescein tagged in the 5′ end. This labeled primer is then extended by T7 DNA polymerase in four different reaction wells containing the four different dideoxynucleotides. The four reaction products are then analyzed by electrophoresis in an ALF DNA Sequencer (Pharmacia Biotech) on polyacrylamide gel.

Computer support for DNA sequence analysis

For searches in DNA sequence databases, multiple sequence analysis and other computer operations on sequences, the Wisconsin Sequence Analysis Package was used (GCG version 8, Unix, Genetics Computer Group, Inc., Madison, Wisconsin, USA).

Sequences for 16S rRNA genes were retrieved for *R. dentocariosa*, accession number M59055 (EMBL/GenBank), 1476 bp, and for the closely related *Stomatococcus mucilaginosus*, accession number X87758 (EMBL/GenBank), 1467 bp. The sequence for the 16S rRNA gene of *Escherichia coli* was used to position the nucleotides showing variations in the corresponding gene of *R. dentocariosa* [29].

RESULTS

Species identification using 16S rRNA gene sequencing

When partial 16S rRNA gene sequences obtained from the 10 strains were compared with sequence databases using the Fasta (GCG) or the Blast programs, all sequences showed the highest similarity with the 16S rRNA gene from *R. dentocariosa* (Table 3). Strains CCUG 32449 (strain no. 3, 407 bp), CCUG 30072 (strain no. 1, 315 bp), and CCUG 33996 (strain no. 2, 265 bp) showed complete identity (100%) with *R. dentocariosa* when compared with the complete EMBL nucleotide database. The second-ranking identity for the longest sequence (CCUG 32449, 407 bp) was *Stomatococcus mucilaginosus*, with an identity level of 96.3%. Similarity levels then went down to around 94% for other species and then leveled off. The results showed that the three isolates belonged to the species *R. dentocariosa*.

Table 3 Partial 16S rRNA gene sequences of *Rothia dentocariosa* strains from CCUG compared with the type strain sequence of *R. dentocariosa*, ATCC 17931

No.	CCUG number	Length of sequence	Sequence identity (%)	*R. dentoceriosa* subspecies
1	30072	315 bp	100	I
2	33996	265 bp	100	I
3	32449	407 bp	100	I
4	28595	296 bp	99.7	I
5	30754	508 bp	98.4	II
6	27465	322 bp	99.7	II
7	33543	368 bp	98.6	II
8	17835	456 bp	98.5	II
9	35343	515 bp	98.3	II
10	25688	556 bp	99.1	II

When CCUG 25688 sequence results (strain no. 10, 556 bp) were similarly compared with the EMBL nucleotide database, the highest similarity level was also noted for the small-subunit rRNA gene of *R. dentocariosa*, at 99.1%, and with the second species in rank, also *Stomatococcus mucilaginosus*, at 97.1%. The six other strains in the group all showed a highest similarity with *R. dentocariosa*, with *Stomatococcus mucilaginosus* as the second closest for most sequences. Also for these strains the results indicated that they belonged to the species *R. dentocariosa*.

Definition of two species groups within *Rothia dentocariosa*

Similarity levels for the 10 strains when compared with the *R. dentocariosa* type strain sequence (M59055) reached the expected 100% agreement for only three strains (Table 3).

When the sequences were further studied for discrepancies at the nucleotide level, the 10 CCUG strains could be divided into two distinct groups. One group consisted of four isolates with the highest degrees of similarity with the reference strain (Tables 3 and 4). There was an overall agreement in sequence between these two strains. The other group, however, showed a consistent pattern in their nucleotide sequences at four distinct positions in the variable V7 region, different from the reference sequence (Tables 3 and 4). T was replaced by C at position 597, C by T at position 608, T by C at position 610, and G by A at position 684 (position numbers according to *R. dentocariosa* reference sequence M59055, EMBL/GenBank) (Table 4). The results obtained showed a clear separation of genomovar I strains consisting of regular *R. dentocariosa* strains from a majority of similar strains with a

Table 4 Genetic polymorphism in the V7 region of the 16S rRNA gene of *Rothia dentocariosa*

Type	V7 sequence, nr–nr	*R. dentocariosa* strains
I	5′-TGGGGCTTAACCCTG G-3′(612)...5′-G-3′(684)	1, 2, 3, 4 (Table 3)
II	5′-CGGGGCTTAACTCCG G-3′(612)...5′-A-3′(684)	5, 6, 7, 8, 9, 10 (Table 3)
Escherichia coli	5′-CCGGGCTCAAC CTGGG-3′(2145)..5′-G-3′	

The sequences shown range from position 597 to 612 in the V7 region with changes in positions 597, 608 and 610, and also in position 684 with a base switch as shown for the two genetic types. Sequence positions refer to the 16S rRNA gene numbering of the *Rothia dentocariosa* type strain ATCC 17931 (acc. no. M59055). The corresponding nucleotide positions in the *Escherichia coli* gene are 2130, 2141, 2143, and 2217 (acc. no. J01695) and are therefore located within the variable V7 region of the small ribosomal subunit. The sequences are aligned for maximal fit, creating a gap in each sequence located at the same position for the *Rothia dentocariosa* strains as indicated.

different 16S rRNA gene sequence, genomovar II. The differences in as many as four positions in the V7 part of the gene indicate that these six strains belong to a new species within the genus *Rothia*.

Whole cell protein analysis (SDS-PAGE) and cluster analysis

When the 10 *R. dentocariosa* strains, as well as 31 other representatives of various related species, including three additional *R. dentocariosa* strains, were subjected to whole cell protein analysis followed by numerical cluster analysis, the strains showed some interesting patterns (Figure 1). The *R. dentocariosa* strains segregated into two different clusters with a similarity between the two groups of less than 60% ($\approx$56%). In the first cluster, all four strains belonging to 16S rRNA genomovar I were found (Figure 1; Table 3). These strains represent the true *R. dentocariosa* species. In the second cluster, all six strains belonging to 16S rRNA genomovar II were found (Figure 1; Table 3). It is therefore quite clear that the segregation into two genetic groups by 16S rRNA gene sequencing (Table 4) is in agreement with that obtained from whole cell protein profile analysis, an independent method of strain characterization. The levels of differences seen with protein profiling are of such a magnitude as to suggest the existence of two different species among isolates presently identified as *R. dentocariosa*.

Microbiological characterization and biochemical profile

The 10 *R. dentocariosa* isolates grew on McLeod plates in a CO_2 atmosphere to form grayish colonies, appearing wet and with a pearly to whitish look, in 1–2 days. On 5% horse blood Columbia agar in a CO_2 atmosphere the colonies were gray to translucent, often growing down into the agar with a dry appearance and creating a slight indentation in the agar surface.

The 10 strains identified as *R. dentocariosa* by 16S rRNA gene sequencing as shown above were tested using the API Coryne commercial identification kit. Eight of the strains gave the API code 7050125, one strain 7050121 (no. 3) and another one 6010121 (no. 9). The catalase test was negative for these two latter strains, and this was confirmed in separate catalase tests. As to the reading of the results, the pyrazinamidase reaction was weak but definitely positive.

When results of API Coryne for the 10 strains were analyzed in IDBact using the API Coryne Plus matrix, the profiles 7050125 and 7050121 gave correct identification of *R. dentocariosa*, with identification scores of 0.999. The second choice was *Corynebacterium kutscheri*, but with identification scores below 0.0003 and 0.00002, respectively. For the third API Coryne result, 6010121, the identification was correctly *R. dentocariosa*, but with an identification score of 0.897 and with *Arcanobacterium haemolyticum* as the second choice, with an identification score of 0.082, a 10-fold difference in probability. Using the IDBact program and the extended API Coryne Plus matrix, it is therefore possible to correctly identify clinical isolates of *R. dentocariosa*. The two catalase-negative strains belonged to 16S rRNA gene types I and II, respectively, and this feature therefore does not correlate with genetic type.

The 10 strains of *R. dentocariosa* were also tested using the API 20 Strep biochemical identification kit. All strains yielded an API code of 5140010. According to the API identification system, this code is rejected as 'identification not valid'. The very same code is obtained for *Stomatococcus mucilaginosus* strains [15].

Antibiotic susceptibility of *Rothia dentocariosa* strains

The 10 strains of *R. dentocariosa* were tested in disk diffusion experiments for susceptibility to penicillin G, penicillin V, ampicillin, piperacillin, cefuroxime, cefotaxime, imipenem, gentamicin, tetracycline, erythromycin, clindamycin, chloramphenicol, rifampicin, vancomycin, and ciprofloxacin. All strains showed inhibition zones around the different antibiotic disks with diameter values consistent with a biologically homogeneous population of strains. For all antibiotics and strains tested, the inhibition zones were in the range of the susceptible (S), using species-related breakpoints [25,30] for Gram-positive aerobic cocci other than *Staphylococcus* and *Enterococcus* [21]. The

mean zone diameter values were as follows: penicillin G 51 mm, penicillin V 52 mm, ampicillin 50 mm, piperacillin 45 mm, cefuroxime 47 mm, cefotaxime 47 mm, imipenem 49 mm, gentamicin 31 mm, tetracycline 35 mm, erythromycin 43 mm, clindamycin 31 mm, chloramphenicol 39 mm, rifampicin 41 mm, vancomycin 26 mm, and ciprofloxacin 28 mm.

Patient information

A summary of the clinical findings in the patients yielding the *R. dentocariosa* isolates is given in Table 1. The age of the patients ranged from 30 to 63 years; there were three females and five males. No clinical data were available for two of the cases. The specimens were mostly blood samples (five), but there was one pharynx, one peritoneal dialysate fluid and one mitral valve sample. Patient no. 4 with a positive *R. dentocariosa* culture from blood also gave a positive culture from a heart valve at autopsy. Three of the strains were isolated from patients with endocarditis, all male. One of these patients died but the other two, aged 43 and 59, recovered with antibiotic therapy. Exact records of the drugs given were not available.

DISCUSSION

The use of 16S rRNA gene sequencing for speciation of a group of 10 bacterial isolates was expected to provide a straightforward identification as part of our routine procedures. However, when the sequence results of the 10 strains were analyzed in detail, they revealed a division of the species *R. dentocariosa* into two separate groups, genomovar I, regular *R. dentocariosa*, and genomovar II (Tables 3 and 4). Moreover, when the protein profiles of the 10 strains were compared, a similar segregation into two groups was obtained (Figure 1). The two methods are independent of each other in terms of study parameters, one method revealing sequence differences at the gene level for ribosomal RNA, and the other revealing minor or major differences in proteins coded for by many different genes. Such a concordance between two completely independent methods of identifying two different groups of *R. dentocariosa* species suggests fundamental differences between these two groups. It has also been shown in several studies that the results of whole-organism protein electrophoresis analysis correlate well with those of DNA–DNA hybridization experiments, i.e. strains with very similar whole cell protein patterns share high DNA–DNA hybridization values [31,32].

Most reports and descriptions of *R. dentocariosa* suggest the presence of a well-defined and homogeneous bacterial species [17,33–42]. In an early description from 1975 [36] there were four biotypes described as well as two serotypes of *R. dentocariosa*. There were no indications, however, that these different phenotypes would reflect any basic differences on a subspecies level. Another extensive numerical taxonomic study of 222 strains, including seven strains of *R. dentocariosa*, indicated some heterogeneity [42], but the division into two types was rather arbitrary and more based on at what level a segregation is recognized. In a recent article by Sutcliffe et al [43] the application of pyrolysis mass spectrometry to 18 strains identified as *R. dentocariosa* revealed a heterogeneity, with three strains definitely outside the species and the remaining ones segregating into two groups with an additional strain as an outsider. This is a clear indication of species heterogeneity. More compelling evidence for a subdivision of *R. dentocariosa* into more than one species was provided by Fotos et al using protein profile analysis [44]. They identified one group including the *R. dentocariosa* reference strain, another one showing different biochemical, serotype and protein profile results and a third group comprising a small and slightly heterogeneous collection. Their group 2 might very well correspond to our proposed new *Rothia* species, genomovar II, a possibility which will be explored in the future.

The proposition of this paper following the results dividing *R. dentocariosa* strains into two separate groups is to suggest genomovar I *R. dentocariosa sensu stricto*, and genomovar II, a new *Rothia* species. The basis for definition of a new species has to include complete 16S rRNA gene sequences as well as more extensive biochemical and microbiological tests. It might also require DNA–DNA hybridization experiments, since 16S rRNA gene sequencing has been questioned as a reliable basis for discrimination between closely related species [45]. Degrees of 16S rRNA gene similarity between species vary. Among streptococci, for example, established and recognized species show levels of similarities between different species as high as 99.3%, 99.1%, 98.8% and 98.7% [46]. The differences seen between the two types of *R. dentocariosa* might therefore very well be in agreement with the presence of two different species, where the present genomovar I would correspond to the established species *R. dentocariosa*, and genomovar II could represent a new *Rothia* species closely related to *R. dentocariosa* as well as to *Stomatococcus mucilaginosus*.

The DNA base differences detected between *R. dentocariosa* genomovars I and II reside in the V7 region which codes for a variable region of the rRNA molecule. The three-dimensional structure of the ribosome is primarily determined by the RNA sequences of the subunits and then stabilized by

ribosomal proteins. The loop structures of rRNA are rather well conserved. Although the present variations between two groups of *R. dentocariosa* strains were found in a variable region, it is of interest to note that the three changes in positions 597, 608 and 610 (Table 4) do not change the number of loop interactions as compared to the structure seen in the reference rRNA structure of *Escherichia coli*, with five strong bonds and one weak nucleotide interaction in the loop.

R. dentocariosa has been described since 1979 as a rare cause of endocarditis and also of other serious infections [1–14]. It might therefore be considered rather surprising that strains of this species were not identified in individual laboratories, including our own, but sent to a reference laboratory for speciation. It is even more surprising that neither CCUG nor two other reference laboratories in other countries (personal communications) succeeded in directly identifying strains belonging to this group to the proper species level. All these events are therefore clear indications that the awareness of *R. dentocariosa* or other *Rothia species* as human pathogens is quite low among clinical microbiologists. One additional indication of this is the fact that API Coryne does not include *R. dentocariosa* in the identification matrix.

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References

1. Sudduth EJ, Rozich JD, Farrar WE. *Rothia dentocariosa* endocarditis complicated by perivalvular abscess. Clin Infect Dis 1993; 17: 772–5.
2. Anderson MD, Kennedy CA, Walsh TP, Bowler WA. Prosthetic valve endocarditis due to *Rothia dentocariosa*. Clin Infect Dis 1993; 17: 945–6.
3. Weersink AJL, Rozenberg-Arska M, Westerhof PW, Verhoef J. *Rothia dentocariosa* endocarditis complicated by an abdominal aneurysm. Clin Infect Dis 1994; 18: 489–90.
4. Ruben SJ. *Rothia dentocariosa* endocarditis. Western J Med 1993; 159(6): 690–1.
5. Isaacson JH, Grenko RT. *Rothia dentocariosa* endocarditis complicated by brain abscess. Am J Med 1988; 84: 352–4.
6. Broeren SA, Peel MM. Endocarditis caused by *Rothia dentocariosa*. J Clin Pathol 1984; 37: 1298–300.
7. Schands JW. *Rothia dentocariosa* endocarditis. Am J Med 1988; 85: 280–1.
8. Schafer FJ, Wing EJ, Norden CW. Infectious endocarditis caused by *Rothia dentocariosa*. Ann Intern Med 1979; 91(5): 747–8.
9. Pape J, Singer C, Kiehn TE, Lee BJ, Armstrong D. Infective endocarditis caused by *Rothia dentocariosa*. Ann Intern Med 1979; 91(5): 746–7.
10. Nivar-Aristy RA, Krajewski LP, Washington JA. Infection of an arteriovenous fistula with *Rothia dentocariosa*. Diagn Microbiol Infect Dis 1991; 14: 167–9.
11. Lutwick LI, Rockhill RC. Abscess associated with *Rothia dentocariosa*. J Clin Microbiol 1978; 8: 612–13.
12. Pers C, Kristiansen JE, Joensson V, Hansen NE. *Rothia dentocariosa* septicaemia in a patient with chronic lymphocytic leukaemia and toxic granulocytopenia. Dan Med Bull 1987; 34: 322–3.
13. Minato K, Abiko Y. β-Lactam antibiotics resistant *Rothia dentocariosa* from infected postoperative maxillary cyst: studies on R-plasmid and β-lactamase. Gen Pharmacol 1984; 15(4): 287–92.
14. Schiff MJ, Kaplan MH. *Rothia dentocariosa* pneumonia in an immunocompromised patient. Lung 1987; 165: 279–82.
15. Granlund M, Linderholm M, Norgren M, Olofsson C, Wahlin A, Holm SE. *Stomatococcus mucilaginosus* septicemia in leukemic patients. Clin Microbiol Infect 1996; 2: 179–85.
16. Funke G, Hutson RA, Bernard KA, Pfyffer GE, Wauters G, Collins MD. Isolation of *Arthrobacter* spp. from clinical specimens and description of *Arthrobacter cumminsii* sp. nov. and *Arthrobacter woluwensis* sp. nov. J Clin Microbiol 1996; 34(10): 2356–63.
17. Funke G, von Graevenitz A, Clarridge III JE, Bernard KA. Clinical microbiology of coryneform bacteria. Clin Microbiol Rev 1997; 10(1): 125–59.
18. Vemelen K, Mertens I, Vandeven J, Verhaegen J, Verbist L. Bacteraemia with *Leptotrichia buccalis*: report of a case and review of the literature. Acta Clin Belg 1996; 51(4): 265–70.
19. Edmond MB, Riddler SA, Baxter CM, Wicklund BM, Pasculle AW. *Agrobacterium radiobacter*: a recently recognized opportunistic pathogen. Clin Infect Dis 1993; 16(3): 388–91.
20. Ericsson HM, Sherris JC. Antibiotic sensitivity testing. Report of an International Collaborative Study. Acta Pathol Microbiol Scand B 1971; Suppl 217: 1–90.
21. Swedish Reference Group for Antibiotics. Antimicrobial susceptibility testing of bacteria. Stockholm: National Bacteriological Laboratory, 1990.
22. Bjorklind A, Ringertz S, Kronvall G. Types of interpretive errors in susceptibility testing. APMIS 1989; 97: 941–8.
23. Kronvall G. Analysis of a single reference strain for determination of gentamicin regression line constants and inhibition zone diameter breakpoints in quality control of disk diffusion antibiotic susceptibility testing. J Clin Microbiol 1982; 16(5): 784–93.
24. Ringertz S, Olsson-Liljequist B, Kronvall G. Antimicrobial susceptibility testing of *Haemophilus influenzae*. Improvement of accuracy of the disk diffusion test. J Antimicrob Chemother 1990; 26: 479–89.
25. Kronvall G, Ringertz S. Antibiotic disk diffusion testing revisited. Single strain regression analysis. APMIS 1991; 99: 295–306.

26. Pot B, Vandamme P, Kersters K. Analysis of electrophoretic whole-organism protein. In: Goodfellow M, O'Donnell AG, eds. Modern microbial methods. Chemical methods in prokaryotic systematics. Chichester: Wiley and Sons, 1994; 493–521.
27. Pettersson B, Uhlén M, Johansson K-E. Phylogeny of some ruminant mycoplasmas based on 16S rRNA sequences and definition of a new cluster within the hominis group. Int J Syst Bacteriol 1996; 46: 1093–8.
28. Pettersson B. Direct solid-phase 16S rDNA sequencing: a tool in bacterial phylogeny. Stockholm: Department of Biochemistry and Biotechnology, The Royal Institute of Technology, 1997.
29. Brosius J, Dull TJ, Sleeter DD, Noller HF. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J Mol Biol 1981; 148: 107–27.
30. Kronvall G, Ringertz S, Karlsson I, Göransson E, Dornbusch K. Laboratory- and species-specific interpretive breakpoints for disk diffusion tests of chloramphenicol susceptibility of *Haemophilus influenzae*. Antimicrob Agents Chemother 1988; 32(10): 1484–9.
31. Vandamme P, Pot B, Falsen E, Kersters K, Devriese LA. Taxonomic study of Lancefield streptococcal groups C, G, and L (*Streptococcus dysgalactiae*) and proposal of *S. dysgalactiae* subsp. *equisimilis* subsp. nov. Int J Syst Bacteriol 1996; 46: 774–81.
32. Vandamme P, Devriese LA, Pot B, Kersters K, Melin P. *Streptococcus difficile* is a nonhemolytic group B, type Ib *Streptococcus*. Int J Syst Bacteriol 1997; 47: 81–5.
33. Stackebrandt E, Koch C, Gwozdiak O, Schumann P. Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. Int J Syst Bacteriol 1995; 45(4): 682–92.
34. Bernard K, Bellefeuille M, Hollis DG, Daneshvar MI, Moss CW. Cellular fatty acid composition and phenotypic and cultural characterization of CDC fermentative coryneform groups 3 and 5. J Clin Microbiol 1994; 32(5): 1217–22.
35. Holmberg K, Hallander HO. Numerical taxonomy and laboratory identification of *Bacterionema matruchotii*, *Rothia dentocariosa*, *Actinomyces naeslundii*, *Actinomyces viscosus*, and some related bacteria. J Gen Microbiol 1973; 76: 43–63.
36. Slack JM, Gereneser MA. Anonymous Actinomyces and filamentous bacteria. Biology and pathogenicity. Minneapolis, Minnesota, USA: Burgess Publishing Company, 1975: 71–8.
37. Noble WC, Dixon JMS, Parker MT, Duerden BI, eds. Systematic bacteriology (Topley & Wilson's), Vol 2, 8th edn. London: Edward Arnold, 1990: 103–18.
38. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST, eds. Bergey's manual of determinative bacteriology, 9th edn. Baltimore: Williams & Wilkins, 1994: 571–96.
39. Clarridge JE, Spiegel CA, Murray PR, et al, eds. Manual of clinical microbiology, 6th edn. Washington, DC: ASM Press, 1995: 357–78.
40. Kalfas S, Edwardsson S. Identification procedures for oral *Actinomyces* species. Oral Microbiol Immunol 1990; 5: 39–42.
41. Jones D, Collins MD, Sneath PHA, Mair NS, Sharpe ME, Holt JG, eds. Bergey's manual of systematic bacteriology. Baltimore: Williams & Wilkins, 1986: 1261–434.
42. Schoefield GM, Schaal KP. A numerical taxonomic study of members of the *Actinomycetaceae* and related taxa. J Gen Microbiol 1981; 127: 237–59.
43. Sutcliffe IC, Manfio GP, Schaal KP, Goodfellow M. An investigation of the intra-generic structure of *Rothia* by pyrolysis mass spectrometry. Zentral Bakteriol 1997; 285: 204–11.
44. Fotos PG, Gerencser MA, Yelton DB. Strain differentiation of *Rothia dentocariosa* and related isolates by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Int J Syst Bacteriol 1984; 34(2): 102–6.
45. Stackebrandt E, Goebel BM. Taxonomic note: A place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 1994; 44(4): 846–9.
46. Bentley RW, Leigh JA, Collins MD. Intrageneric structure of *Streptococcus* based on comparative analysis of small-subunit rRNA sequences. Int J Syst Bacteriol 1991; 41(4): 487–94.