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Phylogeny of the genus *Simonsiella* and other members of the *Neisseriaceae*

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16S rDNA was sequenced from 16 strains of the oral commensal *Simonsiella* and was used to assess relationships between *Simonsiella* strains and other members of the *Neisseriaceae*. In all analyses, *Simonsiella* strains grouped according to established species designations and the mammalian hosts from which they were isolated. The commensals from cats and dogs formed a monophyletic group. The monophyly of the genus *Simonsiella*, however, could be neither supported nor rejected; deep nodes in the trees were unstable depending on the phylogenetic method or on the particular sequences used in the analysis. Instabilities may be attributable to frequent gene transfer between *Neisseria* or other members of the *Neisseriaceae* and *Simonsiella*.

Keywords: *Simonsiella*, phylogeny, *Neisseriaceae*, horizontal gene transfer

INTRODUCTION

Simonsiella is a morphologically unique oral commensal of mammals. Individual cells of *Simonsiella* are wide (1·9–6·4 μm), short (0·5–1·3 μm) and relatively flat (0·5–1·3 μm), and attach to form monoseriate filaments that are 8–12 cells long. Filaments are bent slightly to form a watchband-like shape and show dorsal–ventral asymmetry. The ventral surface is covered with thin filaments that protrude at right angles from the cells (Pangborn *et al*., 1977). This surface attaches to and glides on epithelial cells in the oral cavity and upper respiratory tract of the host.

Simonsiella-like bacteria have been reported in many animals including humans, horses, cows, pigs, sheep, dogs, rabbits, cats, guinea pigs and chickens (Kuhn, 1981). However, few studies have resulted in the isolation and detailed study of axenic *Simonsiella* cultures. A notable exception was the work by Kuhn *et al*. (1978), which entailed the collection of over 50 *Simonsiella* strains from humans, sheep, dogs and cats. A numerical taxonomic study showed that most of the *Simonsiella* strains grouped according to the mammalian host from which they were isolated. *Simonsiella* isolates from humans and sheep were each monophyletic in a dendrogram derived from the numerical taxonomy data. Isolates from cats and dogs tended to cluster together with strains from the same host; however, neither group was strictly monophyletic based on phenotypic data. These observations led to the proposal that each of these mammals has a unique type of *Simonsiella* (Kuhn *et al*., 1978). The authors suggested that these host groups represented ecospecies: species of bacteria that each occupied a niche in a unique ecosystem, the mouths of different animals. Accordingly, three of the *Simonsiella* groups were assigned to separate species. *Simonsiella muelleri*, *Simonsiella crassa* and *Simonsiella steedae* were proposed for *Simonsiella* strains native to humans, sheep and dogs, respectively; however, the cat *Simonsiella* isolates remained unnamed.

The higher-order taxonomy of *Simonsiella* has been a confusing subject. Steed (1962) designated the family *Simonsiellaceae* to include *Simonsiella* and the superficially similar genus *Alysiella*; however, the genus *Alysiella* has been reported to be unrelated to *Simonsiella* (Stackebrandt *et al*., 1988). Dewhirst *et al*. (1989) sequenced 16S rRNA from the type strain of *S. muelleri* and found that it clustered within the *Neisseriaceae* in the *β-Proteobacteria*. Appropriately, the authors emended the family *Neisseriaceae* to include *Simonsiella*.

Since only one *Simonsiella* strain has been included in published molecular phylogenetic studies, and those studies were restricted to distance analyses that were

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The GenBank/EMBL/DDBJ accession numbers for the *Simonsiella* 16S rDNA sequences reported in this study are AF328141–AF328156.

Table 1. Sources, 16S rDNA accession numbers and references for strains and 16S rDNA sequences used in the study

Culture collections are abbreviated as: Bangor, A. J. Howard, Ysbyty Gwynedd, Bangor LL57 2PW, UK; CCUG, Dept of Clinical Microbiology, University of Göteborg, Göteborg, Sweden; CDC, Centers for Disease Prevention and Control, Atlanta, GA, USA; FDC, Forsyth Dental Center, Boston, MA, USA; LCDC, Laboratory Centre for Disease Control, Ottawa, Canada K1A 0L2; NCTC, L. R. Hill, National Collection of Type Cultures, London NW9 5EQ, UK; NRL, *Neisseria* Reference Laboratory, US Public Health Service Hospital, Seattle, WA 98114, USA.

Strain	Accession no.	Reference
Escherichia coli	J01695	Brosius *et al.* (1978)
Eikenella corrodens		
ATCC 23834^T	M22512	Dewhirst *et al.* (1989)
FDC 373	M22513	Dewhirst *et al.* (1989)
FDC 1073	M22515	Dewhirst *et al.* (1989)
Eikenella sp. CCUG 28283	L06165	Dewhirst *et al.* (1989)
Kingella denitrificans		
ATCC 33394^T	M22516	Dewhirst *et al.* (1989)
CCUG 28284 (= UB-294)	L06166	Dewhirst *et al.* (1993)
Kingella kingae ATCC 23330^T (= Y5^T)	M22517	Dewhirst *et al.* (1989)
Kingella oralis ATCC 51147^T (= CCUG 30450^T)	L06164	Dewhirst *et al.* (1993)
Neisseria animalis ATCC 19573^T (= C3^T)	L06172	Dewhirst *et al.* (1993)
Neisseria canis ATCC 14687^T (= D1^T = D1a^T)	L06170	Dewhirst *et al.* (1993)
Neisseria cinerea ATCC 14685^T (= F1^T = NCTC 10294^T)	AJ239299	Smith *et al.* (1999)
Neisseria denitrificans ATCC 14686^T (= H1^T = H1a^T)	L06173	Dewhirst *et al.* (1993)
Neisseria elongata ATCC 25295^T	L06171	Dewhirst *et al.* (1993)
Neisseria elongata	–	F. E. Dewhirst and others, personal communication
Neisseria flavescens ATCC 13120^T	L06168	Dewhirst *et al.* (1993)
Neisseria gonorrhoeae		
76993	AF146369	D. Raoult and others, personal communication
ATCC 19424^T (= NCTC 83785^T = A59^T = A59a^T)	X07714	Rossau *et al.* (1990)
Neisseria lactamica		
LCDC 77-143 (= L17)	AJ239313	Smith *et al.* (1999)
LCDC 845 (= L19)	AJ239296	Smith *et al.* (1999)
Neisseria macacae ATCC 33926^T (= W1^T = W1a^T)	L06169	Dewhirst *et al.* (1993)
Neisseria mucosa Bangor 15 (= M5)	AJ239279	Smith *et al.* (1999)
Neisseria perflava U15	AJ239295	Smith *et al.* (1999)
'*Neisseria pharyngis*' NCTC 4590 (= O4)	AJ239281	Smith *et al.* (1999)
Neisseria polysaccharea ATCC 43768^T (= P5^T = P6^T = CCUG 18030^T)	L06167	Dewhirst *et al.* (1993)
Neisseria sicca		
LCDC R9742 (= Q13)	AJ239292	Smith *et al.* (1999)
ATCC 49276^T (= Q29^T = Q29a^T = NRL 30016^T)	AJ239294	Smith *et al.* (1999)
Neisseria subflava NRL 30017^T (= U37^T = U37a^T)	AJ239291	Smith *et al.* (1999)
Neisseria weaveri CDC 8142	L10738	Andersen *et al.* (1993)
Simonsiella crassa		
ATCC 27504*	AF328141	This study
ATCC 29446	AF328142	This study
ATCC 29447	AF328143	This study
ATCC 29448	AF328144	This study
Simonsiella muelleri		
ATCC 29433	AF328145	This study
ATCC 29441	AF328146	This study
ATCC 29453^T	AF328147	This study
ATCC 29462	AF328148	This study
Simonsiella steedae		
ATCC 27409^T	AF328153	This study
ATCC 29435	AF328154	This study
ATCC 29445	AF328155	This study
ATCC 29457	AF328156	This study

Table 1 (*cont.*)

Strain	Accession no.	Reference
Simonsiella sp.		
ATCC 27381	AF328152	This study
ATCC 29436	AF328149	This study
ATCC 29437	AF328150	This study
ATCC 29465	AF328151	This study

* Strain ATCC 27504 is a second deposition of the type strain of *S. crassa*, ATCC 15533^T.

not evaluated by tree-validation techniques such as bootstrap replications (Dewhirst *et al*., 1989, 1993), several questions concerning *Simonsiella* evolution remain. These questions include: (i) is the genus *Simonsiella* monophyletic?; (ii) is the integrity of the three *Simonsiella* species supported by molecular data?; and (iii) what is the relationship between the *Simonsiella* isolates from cats and the other *Simonsiella* species? To test the current *Simonsiella* species groupings and determine the diversity of the group within the context of other bacteria, 16S rDNA from 16 representative *Simonsiella* strains was sequenced and analysed. In the process, the phylogeny of the *Neisseriaceae* in general was examined.

METHODS

Bacterial strains. *Simonsiella* strains were donated from the American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated on modified trypticase soy medium (ATCC medium 646), which contains (l^{-1}): 17·0 g trypticase (BBL 11921), 3·0 g phytone (BBL 11906), 5·0 g NaCl, 2·5 g K_2HPO_4 and 4·0 g yeast extract. After the medium was autoclaved and cooled to 50 °C, fetal bovine serum was added to a final concentration of 10% (v/v) to supply growth factors. Strain designations, sources and accession numbers are shown in Table 1.

16S rDNA PCR and sequencing. Genomic DNA was isolated using the Instagene kit (Bio-Rad). 16S rDNA was amplified by PCR using bacterial primers (Reysenbach *et al*., 1994) and the following parameters: 32 cycles of 1·5 min at 94 °C, 1 min at 42 °C and 4 min at 72 °C; the last step of the last cycle was extended to 10 min. The product was purified using commercial columns (Ultrafree MC; Millipore) and sequenced using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and 16S rDNA-specific forward and reverse primers (Dyksterhouse *et al*., 1995). Sequence contigs were created manually in the SeqApp data editor (Gilbert, 1992). Reference sequences were obtained from the National Center for Biological Information (NCBI) or from the Ribosomal Database Project (RDP; Maidak *et al*., 1999).

Sequence alignment and phylogenetic analysis. The prealigned sequences from the 'Sequence Alignments' archive of the 'Download' section of the RDP web site were used as an alignment template. *Simonsiella* sequences and sequences obtained from the NCBI were aligned using the RDP 'Sequence Aligner' program in the 'Online Analyses' section of the RDP web site or were aligned manually. The alignment was checked manually in GeneDoc (K. Nicholas & H. Nicholas; http://www.psc.edu/biomed/genedoc/). As recommended by Kishino & Hasegawa (1989) and others, positions containing gaps were removed. However, it is noteworthy that analyses done without removing positions that contained gaps produced results that were similar to those obtained with gaps removed. The final alignment consisted of 1313 positions, encompassing *Escherichia coli* positions 28–1425 (Brosius *et al*., 1978). It consisted of many members of the *Neisseriaceae* (Table 1) and 20 members of the *γ*- and *β*-*Proteobacteria*, which served as outgroup taxa.

For likelihood, an alignment was initially analysed using DNAPARS (Felsenstein, 1993) and MACCLADE (Maddison & Maddison, 1992) to determine empirically the transition to transversion (Ts/Tv) ratio and nucleotide base frequencies. Using DNAPARS, the alignment was analysed (random sequence input order and 10 subreplicates) and the single most parsimonious result was imported into MACCLADE, where the 'State Changes and Stasis' command was used to determine the Ts/Tv ratio of 1·8. This value and the base frequencies were specified in DNAML analyses.

For parsimony and distance analyses, 10 alignments, consisting of randomly chosen taxa from the original alignment, were created. The alignments were analysed using PAUP (Swofford, 1998) and TREECON (Van de Peer & De Watcher, 1994) for parsimony (random input order and 10 subreplicates) and distance (Kimura correction, neighbour-joining) analyses, respectively. The tree in Fig. 1 was projected using TREECON.

RESULTS AND DISCUSSION

Members of the genus *Simonsiella* possess a morphology that is both striking and unique among the *Bacteria*. For this reason, it would seem likely that they would make up a coherent phylogenetic group that excluded other bacteria. To test this hypothesis, the phylogeny of *Simonsiella* and representatives of the genera *Kingella*, *Eikenella* and *Neisseria* was addressed using maximum-likelihood, parsimony and distance methods. However, the resulting phylogeny depended on the phylogenetic method and on the particular taxa that were included. Significantly, bootstrap support values for any particular relationship between *Simonsiella* strains from different hosts were low, except those grouping the cat and dog isolates. Some analyses

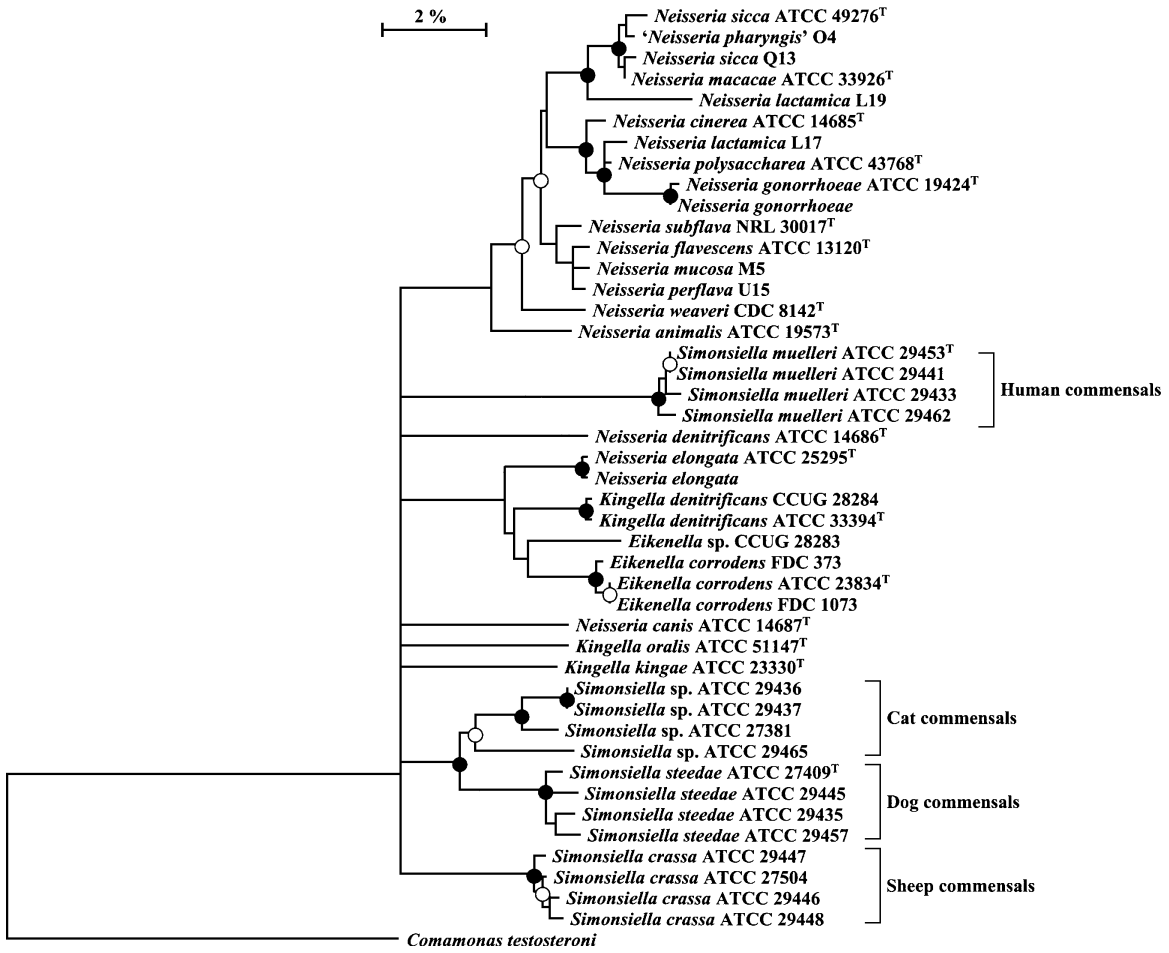

Fig. 1. Consensus neighbour-joining tree. ●, Nodes with > 90 % bootstrap support for all analyses; ○, nodes with > 75 % bootstrap support; nodes with < 50 % support are shown as unresolved. Bar, approx. 2 % nucleotide divergence.

showed *S. muelleri* branching with *Neisseria denitrificans*. A phylogenetic relationship between *S. muelleri* and *N. denitrificans* was supported by relatively high 16S rDNA similarity levels (96·4–96·6 %) and was presented previously by Dewhirst *et al.* (1989, 1993). However, in other analyses, no specific relationship between *S. muelleri* and *N. denitrificans* was implied (data not shown). Bootstrap support for a relationship between *S. muelleri* and *N. denitrificans* was never higher than 65 % (data not shown). Fig. 1 shows a majority-rule tree. Since the *Simonsiella* strains belonged to three lineages whose relationship to each other and to other *Neisseriaceae* lineages is uncertain, the analyses failed to support or reject the hypothesis that the genus *Simonsiella* had a monophyletic origin.

It is possible that the genus *Simonsiella* had a monophyletic origin and that 16S rDNA evidence of the monophyly of the group was lost. Discrepancies abound between 16S rDNA phylogenies of the genus *Neisseria* and those derived from analyses of other loci (Smith *et al.*, 1999) or from chemotaxonomic data (Barrett & Sneath, 1994). Smith *et al.* (1999) suggested that the anomalies were due to interspecies gene exchange. Consistent with this hypothesis, the authors discussed phylogenetic evidence that certain *Neisseria* 16S rDNA sequences are hybrids. Furthermore, it is well known that members of the genus *Neisseria* are competent; documented examples of transformation and recombination between *Neisseria* species are ample in the literature, even when donor and recipient differ by as much as 25 % in their DNA G+C content (Zhou & Spratt, 1992; Feil *et al.*, 1996; Zhou *et al.*, 1997). Thus, members of the genus *Neisseria* could have acquired and recombined with *Simonsiella* 16S rDNA sequences. It is not known whether *Simonsiella* themselves are competent; if they are, then horizontal gene transfer from *Neisseria* or other oral flora to *Simonsiella* could have added to the confusion.

However, it cannot be ruled out that the genus *Simonsiella* is polyphyletic. The ancestor of the *Neisseriaceae* could have been morphologically like modern *Simonsiella*. Loss of *Simonsiella* morphology and gliding motility could have occurred independently several times, giving rise to *Kingella*, *Eikenella* and multiple groups of *Neisseria*. Alternatively, the distinctive *Simonsiella* morphology could have arisen

repeatedly in the *Neisseriaceae*. Indeed, no phenotypic traits of *Simonsiella*, other than morphology and motility, are known to be unique among the *Neisseriaceae*. Given the horizontal gene transfer activity of this family, the question of *Simonsiella* monophyly may never be answered convincingly.

Although the monophyly of the genus *Simonsiella* could not be established, *Simonsiella* groups that corresponded to the mammalian host from which they were isolated were clearly delineated. Three of the four host groups were supported by 100% of bootstrap replications. These corresponded to the existing *Simonsiella* species, *S. muelleri*, *S. steedae* and *S. crassa*, which are respectively commensals of humans, dogs and sheep. The fourth group, comprising *Simonsiella* isolates from domestic cats, was more phylogenetically diverse than the other groups (Fig. 1). Nevertheless, this group was monophyletic in maximum-likelihood analyses and in $\geqslant 82\%$ of all bootstrap replications for distance and parsimony analyses, regardless of the particular taxa that were analysed (data not shown). Thus, our analyses strengthen Kuhn's division of *Simonsiella* into ecospecies. The explanation for this pattern of *Simonsiella* diversity is not clear; however, it is intriguing to speculate whether the divergence of *Simonsiella* into host-specific ecospecies was forced by speciation events of *Simonsiella* hosts. Circumstantial evidence in support of this possibility is that all analyses, phylogenetic (Fig. 1) and phenotypic (Kuhn *et al*., 1978), support a relationship between *S. steedae* and the cat *Simonsiella* isolates. Domestic dogs and cats both belong to the order Carnivora and are thought to have diverged about 45 million years ago (Kumar & Hedges, 1998), whereas the last common ancestors of the carnivores and humans or sheep are thought to have existed 80–100 million years ago (Kumar & Hedges, 1998). Examining phylogenetic relationships between *Simonsiella* isolates from a group of mammals whose evolution is well understood could test the hypothesis that *Simonsiella* co-diverged with their hosts. If a co-evolutionary relationship could be confirmed between *Simonsiella* and its hosts, it would provide a unique opportunity to compare bacterial species with mammalian species that have existed for the same length of time. Such a situation could provide information that could be used to evaluate the current bacterial species concept.

The phylogenetic and ecological distinctness of Kuhn's cat *Simonsiella* isolates suggest that they may represent a distinct species. This notion is also supported by the fact that the cat *Simonsiella* isolates each differ from their closest relatives, members of *S. steedae*, by more than 2·5% in their 16S rDNA sequences (Stackebrandt & Goebel, 1994). However, the most deeply branching of these strains, ATCC 29465 (Fig. 1), did not cluster with the other cat *Simonsiella* isolates in Kuhn's taxonomy study, differing from the others in its ability to reduce nitrate and in containing some fatty acid signatures typical of *S. steedae* (Jenkins *et al*., 1977). Also, the cat *Simonsiella* isolates are phenotypically similar to *S. steedae*. Thus, a decision on the whether a novel species designation is warranted for the cat *Simonsiella* strains will depend on DNA–DNA hybridization data.

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