Application of MLST and Pilus Gene Sequence Comparisons to Investigate the Population Structures of Actinomyces naeslundii and Actinomyces oris

Uta Henssge¹, Thuy Do¹, Steven C. Gilbert¹, Steven Cox¹, Douglas Clark¹, Claes Wickström², A. J. M. Ligtenberg³, David R. Radford¹, David Beighton¹*  
¹Department of Microbiology, The Henry Wellcome Laboratories for Microbiology and Salivary Research, King’s College London Dental Institute, London, United Kingdom, ²Department of Oral Biology, Faculty of Odontology, Malmö University, Malmö, Sweden, ³Department of Oral Biochemistry, ACTA, Amsterdam, The Netherlands

Abstract

Actinomyces naeslundii and Actinomyces oris are members of the oral biofilm. Their identification using 16S rRNA sequencing is problematic and better achieved by comparison of metG partial sequences. A. oris is more abundant and more frequently isolated than A. naeslundii. We used a multi-locus sequence typing approach to investigate the genotypic diversity of these species and assigned A. naeslundii (n = 37) and A. oris (n = 68) isolates to 32 and 68 sequence types (ST), respectively. Neighbor-joining and ClonalFrame dendrograms derived from the concatenated partial sequences of 7 house-keeping genes identified at least 4 significant subclusters within A. oris and 3 within A. naeslundii. The strain collection we had investigated was an under-representation of the total population since at least 3 STs composed of single strains may represent discrete clusters of strains not well represented in the collection. The integrity of these sub-clusters was supported by the sequence analysis of fimP and fimA, genes coding for the type 1 and 2 fimbriae, respectively. An A. naeslundii subcluster was identified with both fimA and fimP genes and these strains were able to bind to MUC7 and statherin while all other A. naeslundii strains possessed only fimA and did not bind to statherin. An A. oris subcluster harboured a fimA gene similar to that of Actinomyces odontolyticus but no detectable fimP failed to bind significantly to either MUC7 or statherin. These data are evidence of extensive genotypic and phenotypic diversity within the species A. oris and A. naeslundii but the status of the subclusters identified here will require genome comparisons before their phylogenic position can be unequivocally established.

Introduction

Actinomyces naeslundii and Actinomyces oris are part of the commensal oral microbiota [1–3], may play a role in pathogenesis of caries [1] and have been isolated from extra-oral infections [4]. The identification of these species has proven difficult over the years since no reliable phenotypic characteristics are known to distinguish between either species [5] though for a period catalase production was used to distinguish between A. naeslundii and isolates then described as human Actinomyces viscosus [6]. Many of these difficulties were resolved by the extensive genetic studies reported by Johnson and colleagues [5] in which A. naeslundii genospecies 1, 2 and WVA 963, on the basis of DNA-DNA hybridization data, were described. Formal species descriptions were not given as they were unable to distinguish between the genospecies with phenotypic tests. However, genotype specific antisera were developed [7] which permitted the identification of A. naeslundii genospecies 1 and 2 from samples of dental plaque and infected dentine associated with dental caries [1]. Subsequent identification of isolates as members of genospecies 1 or 2 have relied on genospecies-specific antisera [7] which has enabled the distribution and phenotypic properties of the two genospecies to be compared. Thus A. naeslundii genospecies 2 isolates were demonstrated to bind to N-acetyl-β-D-galactosamine and acidic proline-rich proteins and to exhibit an N-acetyl-β-D-galactosamine binding specificity signified by N-acetyl-β-D-galactosamine-inhibitable coaggregation with the specified streptococcal strains. A. naeslundii genospecies 1 also bound to N-acetyl-β-D-galactosamine, but commonly not to acidic proline-rich proteins and possessed another N-acetyl-β-D-galactosamine binding specificity to a different set of streptococcal isolates [8]. However, the hemagglutination patterns of strains ascribed to genospecies 1 and 2 were not uniform indicating phenotypic heterogeneity of the surface properties within these taxa. Sequence analysis of the fimA gene, coding for the type 2 fimbriae, from isolates of the two genospecies indicated that there was greater similarity in sequence within genotypes than between genotypes. However, diversity of fimA sequences within each genotype was apparent and strains
LY7 and P-5-N clearly harboured fimA genes with sequences different to those of the other three studied in each genotype although the fimP gene from 5 A. naeslundii genotypes 2 isolates exhibited ≥98 sequence homology. Members of these two species also exhibited different patterns of interaction with other bacteria, red blood cells and salivary proteins [9]. These two species are early colonizers of tooth surfaces but A. naeslundii genotypes 2 is isolated from the mouth more frequently and in greater numbers than A. naeslundii genotypes 1 [5,10].

We have recently reported the descriptions of Actinomyces naeslundii (previously Actinomyces naeslundii genotypes 1), Actinomyces oris (previously Actinomyces naeslundii genotypes 2) and Actinomyces johnsonii (previously Actinomyces naeslundii genotypes WVA963) by means of sequence analysis of housekeeping genes [2]. We found that some isolates identified as genospecies 1 by Hallberg et al [8,10] on the basis of their reactions with genospecies-specific antisera [7] were different to those of the other three studied in each genotype although the fimP gene from each isolate contained ≥98 sequence homology. Members of these two species also exhibited different patterns of interaction with other bacteria, red blood cells and salivary proteins [9]. These two species are early colonizers of tooth surfaces but A. naeslundii genotypes 2 is isolated from the mouth more frequently and in greater numbers than A. naeslundii genotypes 1 [5,10].

We have recently reported the descriptions of Actinomyces naeslundii (previously Actinomyces naeslundii genotypes 1), Actinomyces oris (previously Actinomyces naeslundii genotypes 2) and Actinomyces johnsonii (previously Actinomyces naeslundii genotypes WVA963) by means of sequence analysis of housekeeping genes [2]. We found that some isolates identified as genospecies 1 by Hallberg et al [8,10] on the basis of their reactions with genospecies-specific antisera [7] were different to those of the other three studied in each genotype although the fimP gene from each isolate contained ≥98 sequence homology. Members of these two species also exhibited different patterns of interaction with other bacteria, red blood cells and salivary proteins [9]. These two species are early colonizers of tooth surfaces but A. naeslundii genotypes 2 is isolated from the mouth more frequently and in greater numbers than A. naeslundii genotypes 1 [5,10].

We have recently reported the descriptions of Actinomyces naeslundii (previously Actinomyces naeslundii genotypes 1), Actinomyces oris (previously Actinomyces naeslundii genotypes 2) and Actinomyces johnsonii (previously Actinomyces naeslundii genotypes WVA963) by means of sequence analysis of housekeeping genes [2]. We found that some isolates identified as genospecies 1 by Hallberg et al [8,10] on the basis of their reactions with genospecies-specific antisera [7] were different to those of the other three studied in each genotype although the fimP gene from each isolate contained ≥98 sequence homology. Members of these two species also exhibited different patterns of interaction with other bacteria, red blood cells and salivary proteins [9]. These two species are early colonizers of tooth surfaces but A. naeslundii genotypes 2 is isolated from the mouth more frequently and in greater numbers than A. naeslundii genotypes 1 [5,10].

We have recently reported the descriptions of Actinomyces naeslundii (previously Actinomyces naeslundii genotypes 1), Actinomyces oris (previously Actinomyces naeslundii genotypes 2) and Actinomyces johnsonii (previously Actinomyces naeslundii genotypes WVA963) by means of sequence analysis of housekeeping genes [2]. We found that some isolates identified as genospecies 1 by Hallberg et al [8,10] on the basis of their reactions with genospecies-specific antisera [7] were different to those of the other three studied in each genotype although the fimP gene from each isolate contained ≥98 sequence homology. Members of these two species also exhibited different patterns of interaction with other bacteria, red blood cells and salivary proteins [9]. These two species are early colonizers of tooth surfaces but A. naeslundii genotypes 2 is isolated from the mouth more frequently and in greater numbers than A. naeslundii genotypes 1 [5,10].

We have recently reported the descriptions of Actinomyces naeslundii (previously Actinomyces naeslundii genotypes 1), Actinomyces oris (previously Actinomyces naeslundii genotypes 2) and Actinomyces johnsonii (previously Actinomyces naeslundii genotypes WVA963) by means of sequence analysis of housekeeping genes [2]. We found that some isolates identified as genospecies 1 by Hallberg et al [8,10] on the basis of their reactions with genospecies-specific antisera [7] were different to those of the other three studied in each genotype although the fimP gene from each isolate contained ≥98 sequence homology. Members of these two species also exhibited different patterns of interaction with other bacteria, red blood cells and salivary proteins [9]. These two species are early colonizers of tooth surfaces but A. naeslundii genotypes 2 is isolated from the mouth more frequently and in greater numbers than A. naeslundii genotypes 1 [5,10].

We have recently reported the descriptions of Actinomyces naeslundii (previously Actinomyces naeslundii genotypes 1), Actinomyces oris (previously Actinomyces naeslundii genotypes 2) and Actinomyces johnsonii (previously Actinomyces naeslundii genotypes WVA963) by means of sequence analysis of housekeeping genes [2]. We found that some isolates identified as genospecies 1 by Hallberg et al [8,10] on the basis of their reactions with genospecies-specific antisera [7] were different to those of the other three studied in each genotype although the fimP gene from each isolate contained ≥98 sequence homology. Members of these two species also exhibited different patterns of interaction with other bacteria, red blood cells and salivary proteins [9]. These two species are early colonizers of tooth surfaces but A. naeslundii genotypes 2 is isolated from the mouth more frequently and in greater numbers than A. naeslundii genotypes 1 [5,10].

We have recently reported the descriptions of Actinomyces naeslundii (previously Actinomyces naeslundii genotypes 1), Actinomyces oris (previously Actinomyces naeslundii genotypes 2) and Actinomyces johnsonii (previously Actinomyces naeslundii genotypes WVA963) by means of sequence analysis of housekeeping genes [2]. We found that some isolates identified as genospecies 1 by Hallberg et al [8,10] on the basis of their reactions with genospecies-specific antisera [7] were different to those of the other three studied in each genotype although the fimP gene from each isolate contained ≥98 sequence homology. Members of these two species also exhibited different patterns of interaction with other bacteria, red blood cells and salivary proteins [9]. These two species are early colonizers of tooth surfaces but A. naeslundii genotypes 2 is isolated from the mouth more frequently and in greater numbers than A. naeslundii genotypes 1 [5,10].

We have recently reported the descriptions of Actinomyces naeslundii (previously Actinomyces naeslundii genotypes 1), Actinomyces oris (previously Actinomyces naeslundii genotypes 2) and Actinomyces johnsonii (previously Actinomyces naeslundii genotypes WVA963) by means of sequence analysis of housekeeping genes [2]. We found that some isolates identified as genospecies 1 by Hallberg et al [8,10] on the basis of their reactions with genospecies-specific antisera [7] were different to those of the other three studied in each genotype although the fimP gene from each isolate contained ≥98 sequence homology. Members of these two species also exhibited different patterns of interaction with other bacteria, red blood cells and salivary proteins [9]. These two species are early colonizers of tooth surfaces but A. naeslundii genotypes 2 is isolated from the mouth more frequently and in greater numbers than A. naeslundii genotypes 1 [5,10].

We have recently reported the descriptions of Actinomyces naeslundii (previously Actinomyces naeslundii genotypes 1), Actinomyces oris (previously Actinomyces naeslundii genotypes 2) and Actinomyces johnsonii (previously Actinomyces naeslundii genotypes WVA963) by means of sequence analysis of housekeeping genes [2]. We found that some isolates identified as genospecies 1 by Hallberg et al [8,10] on the basis of their reactions with genospecies-specific antisera [7] were different to those of the other three studied in each genotype although the fimP gene from each isolate contained ≥98 sequence homology. Members of these two species also exhibited different patterns of interaction with other bacteria, red blood cells and salivary proteins [9]. These two species are early colonizers of tooth surfaces but A. naeslundii genotypes 2 is isolated from the mouth more frequently and in greater numbers than A. naeslundii genotypes 1 [5,10].
STs 1–68 were assigned to all *A. oris* and *A. naeslundii* and STs 69–100 were assigned to the *A. naeslundii* strains. A neighbor-joining tree was constructed in MEGA4 [19] using the concatenated sequences of each ST and bootstrap values calculated based on analysis of 1000 resampled datasets.

Further phylogenetic analysis of the sequence data of all *A. oris* and *A. naeslundii* STs was performed using ClonalFrame [20] a technique widely used to assess the evolutionary relationships between strains of the same and closely related bacterial species as it enables bacterial recombination to be taken into consideration when constructing phylogenetic history. The threshold for the consensus tree was set to 0.50 and 0.95. Six ClonalFrame runs were conducted using the default settings, 50,000 iterations which were discarded, followed by 50,000 iterations of which every 100th generation was sampled. Therefore, 501 trees per run were calculated and the data of the six runs were combined and a consensus tree was set to 0.50 and 0.95. Six ClonalFrame runs were conducted using the default settings, 50,000 iterations which were discarded, followed by 50,000 iterations of which every 100th generation was sampled. Therefore, 501 trees per run were calculated and the data of the six runs were combined and a consensus phylogenetic tree was drawn in MEGA4 [19] using the concatenated sequences of each ST and bootstrap values calculated based on analysis of 1000 resampled datasets.

16S rRNA sequence analysis

Partial 16S rRNA gene sequences of selected *A. oris* and *A. naeslundii* isolates identified as outliers to the main species groups were determined. A Neighbor-joining tree was constructed in MEGA4 and bootstrap values calculated based on analysis of 1000 resampled datasets. The tree included the sequences determined here and the sequences for the type strains of *A. viscosus* (X82453), *A. naeslundii* (X81062), *A. johnsonii* (AB343933.1) and *A. oris* (GQ421308).

The fimA and fimP sequences

These sequences were trimmed and edited using BioEdit. The derived sequences were aligned with fimC gene of four *A. naeslundii* genospecies 2 strains (*A. oris* [DQ425102, DQ425099, DQ425101 and AF019629] and three *A. naeslundii* genospecies 1 strains [4.*naeslundii*] [DQ425097, DQ425098 and DQ425100]. The fimA sequence of *A. odontolyticus* strain PK984 (DQ425103) was also included in these analyses as were the fimP gene sequences of three *A. naeslundii* genospecies 2 strains (*A. oris* [AF106053, AF107019 and AF107020], human *A. viscosus* ATCC19249 [AF106034] and the fimA and fimP of *A. oris* MG-1. Neighbor-joining trees were constructed in MEGA4 for the fimA and fimP partial gene sequences and bootstrap values calculated based on analysis of 1000 resampled datasets.

Binding assays

The strains selected for these assays were cultured anaerobically at 37°C on Fastidious Anaerobe Agar (LabM, Bury, UK) supplemented with 5% (v/v) defibrinated horse blood and single colonies transferred to brain-heart infusion broth and grown anaerobically for 2 days. These cultures were used to inoculate Todd Hewett Broth (Oxoid) and grown anaerobically for 2 days. The cells were harvested by centrifugation (2000 g for 10 min), washed and resuspended in 1–3 ml saliva buffer (2 mM KH₂PO₄, 50 mM KCl, 1 mM CaCl₂, 0.1 mM MgCl₂) containing 0.02% NaN₃ and stored at 4°C prior to use.

Parotid saliva was obtained from a volunteer using a sterilized Lashley suction cup placed over the opening of a Stenson’s duct; parotid saliva flow was stimulated by sucking on a sugar-free lemon sweet (Simkins, Sheffield, UK). Ethical permission for the collection of human parotid saliva was obtained from the Ethics Committee of Guy’s and St Thomas Hospital Foundation Trust. Written consent for the collection of the saliva sample was obtained.

A proline-rich protein (PRP)-enriched fraction was obtained from parotid saliva by boiling to remove amylase [21], selective adsorption of statherin with hydroxyapatite [22] and precipitation of histatins with ZnCl₂ [23]. The predominance of PRPs in the resulting preparation was confirmed by SDS-PAGE and Coomassie Blue staining. Gel and sol phases of purified human MUC5B [high molecular weight salivary mucin, MG1] and an

---

**Table 1.** Primers used for the amplification and sequencing of the **fimA** and **fimP** genes.

| PCR target | Primers | Primary primer sequence sets (5’-3’) | Sequencing primers (5’-3’) | bp |
|------------|---------|------------------------------------|---------------------------|----|
| fimA       | fimA-F  (forward) | 1.CCAAGCCCTTTCGGTTG | MACGTCTACCCYAGAAC | 659 |
|            | fimA-R  (reverse) | 1.CGGAAACGACTGCTTG | GCGCTTGCTCVCACCAG | |
| fimP       | fimP-F  | 1.GTGCGACAGACGGACA | CCACGCTACTCCCCAGAA | 679 |
|            | fimP-R  | 1.SACGAGGCAGTAGTAGTCMT | GGGGGCCCTGGTCTC | |

doi:10.1371/journal.pone.0021430.t001
enriched sol fraction of MUC7 (low molecular weight salivary mucin, MG2) were prepared as previously described [24,25]. Protein concentrations were measured using a Pierce BCA kit with a BSA standard (Thermo Scientific). In the case of mucins, the figures were adjusted to take into account their protein percentages [26] for total concentrations.

Saliva and salivary proteins were coated on to MaxiSorb plates (Thermo Scientific) by overnight incubation of dilutions in buffer at 100 μl per well. The PRP fraction were used respectively at concentrations of 4.0, 0.25 and 0.25 μg/ml in 0.1 M NaHCO₃/NaCO₃ buffer, pH 9.6, at 4°C. The MUC5B gel and sol phases and the MUC 7 fraction were used at concentrations of 110, 40 and 1.0 μg/ml respectively in phosphate-buffered saline (PBS) at room temperature. All plates were washed 4× with PBS containing 0.1% Tween 20 (PBS-T). Microwell plates coated with 2.5 μg/ml of human statherin were prepared as previously described [27] except that the statherin was coated by overnight incubation in sodium carbonate buffer (pH 9.6).

For the attachment experiments plates the PRP fraction were prepared in saliva buffer with and without 25 mM lactose [28,29]. With mucin-coated plates the Actinomyces were resuspended in PBS-T at a concentration of approximately 5×10⁸ per ml. The latter buffer was also used for subsequent washing steps with all these types of plate. For the statherin plates, Tris-buffered saline (TBS) containing 1 mM CaCl₂ and 0.1% Tween was used to resuspend bacteria and subsequent washing steps. Duplicate aliquots (100 μl) of the bacterial suspensions were used, the plates were incubated at 37°C for 60 min to allow bacterial attachment, the supernatants decanted and the plates washed 4× with buffer. Attached bacteria were stained [30] by incubation at 37°C for 30 min with 100 μl 0.1 M solution of SYTO 13 (Invitrogen, Paisley, UK). The dye was removed and the fluorescence determined (Labsystems Fluoroskan, Thermo Scientific). To standardize the attachment experiments, initial fluorescence measurements were made with serial dilutions in 100 μl of saliva in the wells of microplates. The bacteria were spun down (2750 g for 30 min at 15°C) and the cells stained with SYTO 13. For subsequent experiments, bacteria were diluted to give a calculated fluorescence of 100 arbitrary units if complete attachment were achieved; in all assays background fluorescence, measured in the

---

**Figure 1.** Neighbor-Joining tree of concatenated sequences of the 7 house-keeping genes of *A. naeslundii* and *A. oris*. Bar is 0.005 substitutions per site. doi:10.1371/journal.pone.0021430.g001
absence of labeled bacteria, was subtracted from test fluorescence values. For each type of plate coating, attachment measurements were made with 3 separate cultures of each strain used.

Results

The 37 A. naeslundii isolates were assigned to 32 unique allelic profiles (STs) while all 68 A. oris isolates yielded unique STs. A neighbor-Joining tree of the concatenated sequences from both species is shown in Fig. 1. The majority of A. naeslundii STs formed a single cluster which contained the A. naeslundii type strain and two additional clusters, AN_1 of 4 STs and AN_2 of 7 STs, were evident supported by bootstrap values of 90% with ST-74 on the periphery of the cluster. A greater level of diversity was found with the A. oris STs in which the majority of STs formed a single cluster which contained the A. oris type strain and addition discrete clusters labeled AO_1 to AO_3 and with ST-56, ST-66 and ST-64 on the periphery of the A. oris clusters. Analysis of the ST data using ClonalFrame produced similar relationships between the A. oris STs (Fig. 2). AO_1 to AO_3 identified but STs 66 and 64, were apparently more closely related to A. naeslundii than to A. oris and ST-56 more closely related to A. oris but still peripheral. The clusters, AN_1 and AN_2, were discrete but ST-74 was within the A. naeslundii cluster containing the type strain of the species.

A neighbor-joining tree based on alignment of the 16S rRNA sequences of the selected STs, those not within the major groupings of the two species, is shown in Fig. 3. Overall the strains originally assigned to either A. oris or A. naeslundii formed two clusters but the bootstrap value supporting this division was not high. However, all the strains in the clusters AN_1 to AN_2 and ST 74 were in a cluster that contained the type strain of A. naeslundii but also the type strain of A. viscosus. When the partial 16S rRNA sequences of the A. oris and A. naeslundii isolates were inspected a region that enabled differentiation between these species was identified (Fig. 4).

Figure 2. 50% majority-rule consensus tree derived using ClonalFrame. Six ClonalFrame runs were conducted using the default settings, the initial 50,000 iterations were discarded and the next 50,000 iterations were sampled at every 100th generation was sampled. Therefore, 501 trees per run were calculated and the data of the six runs were combined and the consensus tree was drawn in MEGA4 [19]. Scale is coalescent units. doi:10.1371/journal.pone.0021430.g002

Figure 3. Neighbor-Joining tree of partial 16S rRNA gene sequences. Approximately 1400 bp sequences of 16S rRNA genes selected A. naeslundii and A. oris strains indicated by strain name and [sequence type] were determined. Sequences of A. naeslundii (X81062.1; ST77), A. viscosus (X82453), A. johnsonii (AB545933.1) and A. oris (GQ421308; ST16) type strains were included for comparative purposes. STs 1–68 are A. oris and 69–100 are A. naeslundii. Only bootstrap values of >60 are shown for clarity. Bar is 0.001 substitutions per site. doi:10.1371/journal.pone.0021430.g003
was AN_3 but which also included ST-66. ST-64 was more closely related to A. oris ATCC 27044, the type strain for the species. The 16S rRNA sequence confirmed the allocation of isolates to A. oris and A. naeslundii on the basis of house-keeping genes. However, analysis of the concatenated sequences of 7 house-keeping genes, by neighbor-joining trees and ClonalFrame analysis, suggested that there existed within both species phylogenetically distinct clusters which might constitute subspecies and this was supported, at least within the A. oris by a comparison of the partial 16S rRNA sequences. To investigate this further, we determined partial sequences of fimA and fimP to determine if the same phylogenetic relationships could be demonstrated using these genes for extracellular proteins. The neighbor-joining tree based on partial fimP sequences is shown in Fig. 6. The A. johnsonii and A. viscosus strains were discrete from those of A. oris and A. naeslundii. Gene fimP was only detected in 3 STs identified as A. naeslundii and these all corresponded to 3 of the 4 STs in AN_1. The other member of AN_1, ST-99, which also harbored a fimA different to that of the other members of AN_1 did not reveal fimP. A single cluster consisting of the STs in AO_1, AO_2 and ST-56 was evident, discrete from the major A. oris cluster. Strains in AO_3 did not harbor fimP. Two STs, 64 and 66, were discrete and on the periphery of the major A. oris cluster.

**Actinomyces binding to salivary proteins**

The A. naeslundii STs 83 (AN_1), 82 and 94 (AN_2) and the A. naeslundii type strain all bound to MUC7 (Fig. 7) but only ST-83 bound to a significant extent to both the PRP preparation and to statherin. A. oris strains ATCC 27044 and 27898 exhibited high binding to both MUC7 and the PRP preparation but only weakly to statherin. ST-42 exhibited high binding to both MUC7, statherin and PRP. The two members of AO_3 examined (STs 44 and 43) exhibited very low binding to all salivary proteins. A. oris ST-41 exhibited high binding to PRP and the PRP preparation. The A. johnsonii type strains exhibited very low binding to all salivary proteins while the A. viscosus type strains exhibited high binding to statherin, no binding to MUC7 and low binding to the PRP preparation. The patterns of binding observed with MUC7 were also apparent with the MUC5B gel and sol preparations. The ability of the selected strains to bind to the salivary proteins, their cluster identity and the presence of fimA and fimP are summarized in Table 2.

---

**Figure 4. 16S rRNA signature differentiating A. naeslundii and A. oris 16S rRNA sequences.** 16S rRNA sequences were aligned using BioEdit [18] and visually compared to detect sequence signatures that differentiated between the species. Figure lists strain names and ST. The first base is base 1010 in 16S rRNA sequence of A. oris ATCC 27044, the type strain for the species. doi:10.1371/journal.pone.0021430.g004
Discussion

The taxonomy of the Actinomyces “viscosus-naeslundii” group has been the subject of much research in order to find valid and reproducible methods to differentiate between these species which were isolated from both humans and rodents. Phenotypic methods to discriminate between these species have proved elusive [5] although for a time *A. viscosus* was, incorrectly, differentiated from *A. naeslundii* on the basis of catalase production [6]. Serological methods were applied to the discrimination of these species [7] but it was not until the extensive taxonomic study of Johnson and colleagues [5] that these serological data could be understood in relation to a definite taxonomy. Thus by applying DNA-DNA homology it was shown that human strains of “viscosus-naeslundii” group were divided in three different species as the DNA homology between them was <70% [5]. The proposed classification was: *A. naeslundii* genospecies 1, *A. naeslundii* genospecies 2 and the separate Actinomyces serotype WVA 963. The animal strain was classified as *A. viscosus*. In that study, these four taxa shared between 26 to 44% DNA relatedness to each other. A slightly higher similarity was found between *A. viscosus* and *A. naeslundii* genospecies 1 (55%). Furthermore, it was shown that within *A. naeslundii* genospecies 2, strains from *A. naeslundii* serotypes II, NV and *A. viscosus* serotype II were closer related to each other (63 to 79%) than to *A. naeslundii* serotype III (51 to 62%). Subsequently, antisera were used in many studies [1,10] to identify *A. naeslundii* from the human oral cavity as genospecies 1 or 2. 16S rRNA sequence analysis was widely used to identify many taxa but this approach was reported previously to be suitable for the discrimination of *A. oris* and *A. naeslundii*. However, our analysis here shows that there are limited but discrete sequence differences between these species and that these human species are separable but with a low bootstrap value. However, we [2] previously used a concatenated gene sequencing approach to discriminate between

![Figure 5. Neighbor-joining tree showing relationships between fimA sequences.](image)

![Figure 6. Neighbor-joining tree showing relationships between fimP sequences.](image)
strains identified as A. naeslundii and were able to amend the description of A. naeslundii (genospecies 1) and described new species A. oris (genospecies 2) and A. johnsonii (WVA 963).

Here we describe some of the divergence present within A. oris and A. naeslundii using an MLST approach supplemented with an analysis of the partial sequences of fimA and fimP genes. Within both species discrete clusters, characterized by high bootstrap values, were identified and a significant proportion of the collection of each species was present in these discrete clusters. Examination of the Neighbor-joining or ClonalFrame dendrograms suggests that the strain collection we had investigated was an under-represented of the total population since, at least, STs 56, 64 and 66 appear to represent discrete clusters of strains not well represented in the collection.

Previously the fimA gene, the major subunit of type-2 fimbriae, was sequenced in four strains for each “A. naeslundii genospecies 1 and 2” revealing conserved regions as well as sequence differences [9]. The fimP gene, major subunit of type-1 fimbriae, was obtained from four strains of “A. naeslundii genospecies 2” and a human “A. viscosus” strain [31]. The fimA and fimP gene sequences are distinct from the housekeeping genes used to construct the neighbor-joining tree. However, the relationships between the STs in the house-keeping gene tree and the fimA tree are very similar. Thus STs in AN_1 and AN_2 form discrete clusters in the fimA tree distinct from the main A. naeslundii cluster although ST-99 [AN_1] harbored a fimA gene indistinguishable from that of the majority of A. naeslundii strains. Similarly with the A. oris strains AO_1, AO_2 and AO_3 formed discrete clusters with fimA although AO_3 formed a cluster with the A. odontolyticus fimA sequence [DQ425103]. This suggests that this gene was acquired by horizontal gene transfer from A. odontolyticus while ST-99 appears to have acquired a fimA gene from a member of the main A. naeslundii cluster supporting our earlier observation of horizontal gene transfer between A. oris and A. naeslundii [32]. The two STs 64 and 66, which on the basis of the neighbor-joining tree were more similar to A. oris while the ClonalFrame analysis suggested they were more closely related to A. naeslundii formed a small cluster adjacent to the A. naeslundii fimA sequences which also included the sequence of P-5-N1 [DQ425097]. The sequence of A. oris ST-41 was closely related to that of A. naeslundii. The A. johnsonii and A. viscosus sequences were discrete while the sequence of ST-56, a lone ST in the neighbor-joining analysis, was also discrete but closely related to the A. oris cluster.

Type-1 fimbriae (fimP) were mainly found in “A. naeslundii genospecies 2” and may be necessary for early colonization of the tooth through binding to the acquired pellicle. They promote protein-protein binding to tooth-adsorbed salivary acidic proline-rich proteins (PRPs) and statherin [33–35]. Bacteria only bind to

---

**Table 2. Summary of the ST binding characteristics.**

| Cluster¹ | fimA² | fimP³ | Binding to: |
|----------|-------|-------|-------------|
|          |       |       | MUC7⁴ | PRP⁵ | Statherin⁶ |
| A. naeslundii |       |       |       |       |           |
| ST-77 [ATCC12104=NCTC 10301] | Main | + | – | 3+ | + | +/– |
| ST-94 | AN_2 | +⁴ | – | 3+ | + | +/– |
| ST-82 | AN_2 | +⁴ | – | 4+ | + | +/– |
| ST-83 | AN_1 | +⁵ | + | 5+ | 4+ | 3+ |
| A. oris |       |       |       |       |           |
| ST-16 [ATCC27044] | Main | + | –⁴ | 2+ | 3+ | + |
| ST-33 | Main | + | –⁴ | 2+ | 3+ | 2+ |
| ST-49 | Main | + | –⁴ | 2+ | 5+ | +/– |
| ST-56 | Lone | +⁴ | +⁵ | 2+ | 4+ | 3+ |
| ST-03 | AO_1 | +⁶ | +⁵ | 3+ | 3+ | 3+ |
| ST-54 | AO_2 | +⁶ | +⁵ | 3+ | 3+ | 3+ |
| ST-67 | AO_1 | +⁶ | +⁵ | 5+ | 5+ | 4+ |
| ST-64 | Lone | +⁶ | +⁵ | 2+ | 1+ | +/– |
| ST-66 | Lone | +⁶ | +⁵ | + | 2+ | +/– |
| ST-44 | AO_3 | +⁶ | +⁵ | +/– | +/– | +/– |
| ST-43 | AO_3 | +⁶ | +⁵ | +/– | +/– | +/– |
| ST-41 | Lone | +⁶ | +⁵ | 2+ | 2+ | +/– |
| A. johnsonii |       |       |       |       |           |
| A. viscosus |       |       |       |       |           |

¹Clusters as identified in Figure 1.
²Within each species the superscripts for fimA and fimP designate different partial fimA or fimP gene sequences.
³Binding scores are an arbitrary range from – to 5+.
⁴Values, were identified and a significant proportion of the collection of each species was present in these discrete clusters.
⁵Here we describe some of the divergence present within A. oris and A. naeslundii using an MLST approach supplemented with an analysis of the partial sequences of fimA and fimP genes. Within both species discrete clusters, characterized by high bootstrap values, were identified and a significant proportion of the collection of each species was present in these discrete clusters. Examination of the Neighbor-joining or ClonalFrame dendrograms suggests that the strain collection we had investigated was an under-represented of the total population since, at least, STs 56, 64 and 66 appear to represent discrete clusters of strains not well represented in the collection.

---

![Image](image_url)
PRPs when the proteins are bound to a surface at which they undergo a conformational change [36]. Furthermore, different “J. naeslundii” genospecies 2” strains exhibited varied binding to either PRPs or statherin [37]. Type-2 fimbriae (fimA) were identified in both J. naeslundii genospecies 1 and 2 [38]. This type of fimbriae binds to β-linked galactose and galactosamine structures [39] and thus facilitates binding to glycoproteins and glycoproteins on surfaces of epithelial cells and bacteria [40,41] as well as to the enamel pellicle [37]. Binding via the type-2 fimbriae is lactose inhibited. fimP is not present in the majority of J. naeslundii strains and here we only detected it in the three strains of AN_1. This sequence was distinct from all other fimP sequences. STs of AO_1 and AO_2 and ST-56 possessed highly similar fimP sequences, distinct from those of the main J. oris cluster, as did the two lone STs 64 and 66. Members of AO_3 were similar to those of the major group of J. oris strains. A. viscosus and A. johnsonii were distinct from all other sequences.

The binding properties of the selected strains overall mirrored the presence of the particular fimbriae genes. Thus, J. naeslundii STs 77, 94 and 82 adhered to well MUC7 while exhibiting only a low ability to bind to the PRP preparation or statherin. J. naeslundii ST-83, which possessed both fimA and fimP gene adhered well to all three salivary preparations. Although all J. oris STs possessed both fimA and fimP a number of different binding profiles were observed. Thus, members of the main J. oris cluster, AO_1 and AO_2 tended to adhere to all substrates, as did ST-56 while both AO_3 strains exhibited very low binding. The lone STs, 41, 64 and 66 exhibited overall low binding to the three saliva preparations as did A. johnsonii but A. viscosus exhibited high binding to statherin as has been previously reported.

In conclusion these data clearly demonstrate considerable population diversity within each of the species J. oris and J. naeslundii and show that there is a phenotypic basis to the clusters generated by the analysis of the concatenated sequences of 7 housekeeping genes. The population structure was congruent with that obtained by an analysis of the fimA and fimP partial gene sequences. It is inappropriate to attempt to assign names to these discrete clusters but these data provide a basis for further study to accurately plot the establishment and distribution of these organisms in the human mouth and perhaps to better understand their role in the health of the oral cavity. The phylogenetic relationships between these clusters may be elucidated by the application of whole genome sequence comparison techniques [42].

Supporting Information

Table S1 Allelic profiles and sequence types of the J. oris and J. naeslundii strains. (DOC)

Author Contributions

Conceived and designed the experiments: UH DB SC. Performed the experiments: UH TD SCG DC. Analyzed the data: DB UH TD SC. Contributed reagents/materials/analysis tools: AL CW. Wrote the paper: DB UH TD DR.

References

1. Bowden GH, Nelette N, Ryding H, Cleghorn BM (1999) The diversity and distribution of the predominant ribotypes of Actinomyces naeslundii genospecies 1 and 2 in samples from enamel and from healthy and carious root surfaces of teeth. J Dent Res 78: 1800–1809.
2. Hensage U, Do T, Radford DR, Gilbert SC, Clark D, et al. (2009) Emended description of Actinomyces naeslundii and descriptions of Actinomyces oris sp. nov. and Actinomyces johnsonii sp. nov., previously identified as Actinomyces naeslundii genospecies 1, 2 and WVA 963. Int J Syst Evol Microbiol 59: 509–516.
3. Li J, Helmerhorst EJ, Leone CW, Troxler RF, Yaskell T, et al. (2004) Identification of early microbial colonizers in human dental biofilm. J Appl Microbiol 97: 1311–1319.
4. Hall V, Tallbot PR, Stubbs SL, Duerrlen BI (2001) Identification of clinical isolates of actinomyces species by amplified 16S ribosomal DNA restriction analysis. J Clin Microbiol 39: 3353–3362.
5. Johnson JL, Moore LV, Kaneko B, Moore VE (1990) Actinomyces gergiae sp. nov., Actinomyces venocis sp. nov., designation of two genospecies of Actinomyces naeslundii, and inclusion of A. naeslundii serotypes II and III and Actinomyces viscosus serotype II in A. naeslundii genospecies 2. Int J Syst Bacteriol 40: 273–286.
6. Ellen RF (1976) Establishment and distribution of Actinomyces viscosus and Actinomyces naeslundii in the human oral cavity. Infect Immun 14: 1119–1124.
7. Puttins EE, Bowden GH (1993) Antigenic relationships among oral Actinomyces isolates, Actinomyces naeslundii genospecies 1 and 2, Actinomyces viscosus, Actinomyces ruminis, and Actinomyces oralis. J Dent Res 72: 1374–1385.
8. Hallberg K, Hammarskjöld KJ, Falsen E, Dahlen G, Gibbons RJ, et al. (1998) Actinomyces naeslundii genospecies 1 and 2 express different binding specificities to N-acetyl-beta-D-galactosamine, whereas Actinomyces odontolyticus expresses a different binding specificity in colonizing the human mouth. Oral Microbiol Immunol 13: 327–336.
9. Drobin M, Hallberg K, Ohman O, Birve A, Persson K, et al. (2006) Sequence analyses of fimbrine subunit FimA proteins on actinomyces naeslundii genospecies 1 and 2 and Actinomyces odontolyticus with variant carbohydrate binding specificities. BMC Microbiol 6: 43.
10. Brafield SR, Lynch E, Brighton D (1998) The isolation of Actinomyces naeslundii from sound root surfaces and root carious lesions. Caries Res 32: 100–106.
11. Wallgren G, Johnson J, Schachtele C (1999) Characterization of Actinomyces with genomic DNA fingerprints and RNA gene probes. J Dent Res 72: 1171–1179.
12. Hallberg K, Holm C, Hammarstrom KJ, Kalfas S, Stromberg N (1998) Rhiotype diversity of Actinomyces with similar intraoral tropism but different types of N-acetyl-beta-D-galactosamine binding specificity. Oral Microbiol Immunol 13: 188–192.
13. Hall V, O’Neill GL, Magee JT, Duerden BI (1999) Development of amplified 16S ribosomal DNA restriction analysis for identification of Actinomyces species and comparison with pyrolysis-mass spectrometry and conventional biochemical tests. J Clin Microbiol 37: 2525–2561.
14. Ximenes-Fryse LA, HaJJiege AD, Martin I, Tanner A, Macuch P, et al. (1999) Identification of oral Actinomyces species using DNA probes. Oral Microbiol Immunol 14: 257–265.
15. Maiden MCJ, Bygraves JA, Feil E, Morelli G, Russell JE, et al. (1998) Multilocus sequence typing. A portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A 95: 3140–3145.
16. Aas JA, Paster BJ, Stokes LN, Olsen I, Deswift FE (2005) Defining the normal bacterial flora of the oral cavity. J Clin Microbiol 43: 5721–5732.
17. Lane DJ (1991) 16S/23S rRNA sequencing. In: Nucleic Acid Techniques in Bacterial Systematics 115–175, Stackebrandt E, Goodfellow M, eds. Chichester: Wiley.
18. Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.
19. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.
20. Diedlot X, Faludi D (2007) Inference of bacterial microevolution using multilocus sequence data. Genetics 175: 1251–1266.
21. Proctor GB, Pramanik R, Carpenter GH, Rees GD (2005) Salivary proteins interact with dietary constituents to modulate tooth staining. J Dent Res 84: 73–78.
22. Jensen JJ, Lamkin MS, Oppenheim FG (1992) Adsorption of salivary proteins to hydroxyapatite: a comparison between whole saliva and glandular secretions. J Dent Res 71: 1569–1576.
23. Flora B, Guzman H, Helmerhorst EJ, Troxler RF, Oppenheim FG (2001) A new method for the isolation of histatins 1, 3 and 5 from parotid secretion using zinc precipitation. Protein Expr Purif 23: 196–206.
24. Wickstrom C, Christersson C, Davies JR, Carlstedt I (2000) Macromolecular organization of saliva: identification of ‘insoluble’ MUC5B assemblies and non-mucin proteins in the gel phase. Biochem J 351: 421–429.
25. Wickstrom C, Hamilton JR, Svensater G (2009) Differential metabolic activity by dental plaque bacteria in association with two preparations of MUC5B mucins in solution and in biofilms. Microbiology 155: 53–60.
26. Levine MJ, Reddy MS, Tabak LA, Loomis RE, Bergey EJ, et al. (1987) Structural aspects of salivary glycoproteins. J Dent Res 66: 436–441.
27. Jelani T, Leito D, Lajtenberg AJM, Nazmi K, Veerman ECI (2009) Identification of salivary components that induce transition of byphae to yeast in Candida albicans. FEBS Lett 589: 1102–1110.
28. Qureshi JV, Gibbons RJ (1981) Differences in the adsorptive behavior of human strains of *Actinomyces viscosus* and *Actinomyces naeslundii* to saliva-treated hydroxyapatite surfaces. Infect Immun 31: 261–266.

29. Cisar JO, David VA, Carl SH, Vatter AE (1984) Exclusive presence of lactose-sensitive fimbriae on a typical strain (WVU43) of *Actinomyces naeslundii*. Infect Immun 46: 453–458.

30. Bosch JA, Veerman ECI, Turkenburg M, Hartog K, Bolscher JGM, et al. (2003) A rapid solid-phase fluorimetric assay for measuring bacterial adherence, using DNA-binding stains. J Microbiol Meth 53: 51–55.

31. Li T, Johansson I, Hay DI, Stromberg N (1999) Strains of *Actinomyces naeslundii* and *Actinomyces viscosus* exhibit structurally variant fimbrial subunit proteins and bind to different peptide motifs in salivary proteins. Infect Immun 67: 2053–2059.

32. Do T, Henssge U, Gilbert SC, Clark D, Beighton D (2008) Evidence for recombination between a sialidase (nanH) of *Actinomyces naeslundii* and *Actinomyces oris*, previously named *Actinomyces naeslundii* genospecies 1 and 2*. FEMS Microbiol Lett 288: 156–162.

33. Clark WB, Beem JE, Nesbitt WE, Cisar JO, Tseng CC, et al. (1989) Pellicle receptors for *Actinomyces viscosus* type 1 fimbriae in *vitro*. Infect Immun 57: 3003–3008.

34. Gibbons RJ, Hay DI (1981) Human salivary acidic proline-rich proteins and statherin promote the attachment of *Actinomyces viscosus* LY7 to apatitic surfaces. Infect Immun 56: 439–445.

35. Gibbons RJ, Hay DI, Cisar JO, Clark WB (1988) Adsorbed salivary proline-rich protein 1 and statherin: receptors for type 1 fimbriae of *Actinomyces viscosus* T14V-J1 on apatitic surfaces. Infect Immun 56: 2990–2993.

36. Gibbons RJ (1989) Bacterial adhesion to oral tissues: a model for infectious diseases. J Dent Res 68: 750–760.

37. Stromberg N, Borén T, Carlén A, Olsson J (1992) Salivary receptors for GalNAc beta-sensitive adherence of *Actinomyces* spp.: evidence for heterogeneous GalNAc beta and proline-rich protein receptor properties. Infect Immn 60: 3278–3286.

38. Cisar JO, Sandberg AL, Mergenhagen SE (1984) The function and distribution of different fimbriae on strains of *Actinomyces viscosus* and *Actinomyces naeslundii*. J Dent Res 63: 393–396.

39. Stromberg N, Karlsson KA (1990) Characterization of the binding of *Actinomyces naeslundii* (ATCC 12104) and *Actinomyces viscosus* (ATCC 19246) to glycoprotein-glycolipids, using a solid-phase overlay approach. J Biol Chem 265: 11251–11258.

40. Brennan MJ, Cisar JO, Vatter AE, Sandberg AL (1984) Lectin-dependent attachment of *Actinomyces naeslundii* to receptors on epithelial cells. Infect Immun 46: 459–464.

41. Stromberg N, Borén T (1992) *Actinomyces* tissue specificity may depend on differences in receptor specificity for GalNAc beta-containing glycoconjugates. Infect Immun 60: 3268–3277.

42. Jolley KA, Maiden MC (2010) BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 11: 585.