Streptococcal taxonomy based on genome sequence analyses

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Abstract
The identification of the clinically relevant viridans streptococci group, at species level, is still problematic. The aim of this study was to extract taxonomic information from the complete genome sequences of 67 streptococci, comprising 19 species, by means of genomic analyses, multilocus sequence analysis (MLSA), average amino acid identity (AAI), genomic signatures, genome-to-genome distances (GGD) and codon usage bias. We then attempted to determine the usefulness of these genomic tools for species identification in streptococci. Our results showed that MLSA, AAI and GGD analyses are robust markers to identify streptococci at the species level, for instance, S. pneumoniae, S. mitis, and S. oralis. A Streptococcus species can be defined as a group of strains that share ≥ 95% DNA similarity in MLSA and AAI, and > 70% DNA identity in GGD. This approach allows an advanced understanding of bacterial diversity.

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Introduction

Bacteria are subjected to numerous forces driving their diversification. As a consequence, different strains of a single bacterial species sometimes have the ability to explore distinct niches, to be pathogenic or non-pathogenic and to present different metabolic pathways. In such a scenario, the identification of bacteria isolates to the species level is a hard task.

Currently, the genus *Streptococcus* comprises 99 recognized species, many of which are associated with disease in humans and animals. The viridans group streptococci (VGS) encompass four phylogenetic clusters: Mitis, Mutans, Salivarius and Anginosus, which are part of the human microbiota, being isolated mainly from the oral cavity, gastrointestinal and genitourinary tracts. The Mitis group currently includes the important pathogen *S. pneumoniae* and 12 other recognized species, *S. australis*, *S. cristanus* (formerly *S. crista*), *S. gordonii*, *S. infantis*, *S. mitis*, *S. oligofermentans*, *S. oralis*, *S. parasanguinis* (formerly *S. parasanguis*), *S. peroris*, *S. pseudo-pneumoniae*, *S. sanguinis* (formerly *S. sanguis*) and *S. sinensis*. The Anginosus group includes three recognized species, *S. anginosus*, *S. constellatus* (including two subspecies *S. constellatus* subsp. *constellatus* and *S. constellatus pharyngis*) and *S. intermedius*, and the Salivarius group includes *S. salivarius*, *S. vestibularis*, and *S. thermophilus*.

Currently, bacterial species are considered to be a group of strains (including the type strain) that are characterized by a certain degree of phenotypic consistency, showing > 70% DNA-DNA hybridization values and over 97% 16S rRNA sequence similarity. Identification of streptococci is based on the current taxonomic standards using a combination of 16S rRNA gene sequence analyses, DNA-DNA hybridization, serologic and phenotypic data; however, they have been strikingly resistant to satisfactory classification, reflected in frequently changing nomenclature. For instance, the 16S rRNA gene sequences of *S. mitis* and *S. oralis* are almost identical (> 99%) to *S. pneumoniae*, making the use of this information alone insufficient to distinguish these species.

Recent studies have used whole genome analysis to determine the taxonomic relationships among bacterial species. In order to determine the robustness of genomic markers in streptococci species delineation, we analyzed a collection of 67 complete genomes. The availability of whole genome sequences of several closely related species, for instance, *S. mitis* - *S. oralis* - *S. pneumoniae*, and *S. salivarius* - *S. thermophilus* - *S. vestibularis*, formed an ideal test case for the establishment of the genomic taxonomy of streptococci.

Material and methods

Genome sequence data

The genomic sequences of 67 streptococci that were publicly available for download by June 2nd, 2011 at the National Center for Biotechnology Information (NCBI) under the project accession number indicated in Table 1 were used in this study. The following analyses were performed according to Thompson et al. (2009) and are briefly described below.

16S rRNA gene sequence analysis and multilocus sequence analysis (MLSA)

The 16S rRNA gene sequences and the gene sequences used for MLSA were obtained from GenBank (http://www.ncbi.nlm.nih.gov). The MLSA approach was based on the concatenated sequences of five house-keeping genes (*aroE*, *ddl*, *gki*, *pheS* and *recA)*. The concatenated sequences were aligned with ClustalX program. The phylogenetic inference was based on the neighbour-joining genetic distance method (NJ) using MEGA5. Distance estimations were obtained according to the Kimura-2-parameter for 16S rRNA gene and MLSA. The reliability of each tree topology was checked by 2000 bootstrap replications.

Average amino acid identity (AAI)

The AAI of all conserved protein-coding genes was calculated as described previously. Conserved protein-coding genes between a pair of genomes were determined by whole-genome pairwise sequence comparisons using the BLASTp algorithm. For these comparisons, all protein-coding sequences (CDSs) from one genome were searched against the genomic sequence of the other genome. The genetic relatedness between a pair of genomes was measured by the AAI of all conserved genes between the two genomes as computed by the BLAST algorithm. By this approach, a value of < 95% AAI of protein-coding genes indicates separate species.

Codon usage

Codon usage bias was calculated for each genome. The effective number of codons used in a sequence (NCe) was calculated using CHIPS (http://emboss.bioinformatics.nl/cgi-bin/emboss/chips) with the default parameters.

Determination of dinucleotide relative abundance values and genomic dissimilarity

Mononucleotide and dinucleotide frequencies were calculated using COMPEQ (http://emboss.bioinformatics.nl/cgi-bin/emboss/comseq) with default parameters. Dinucleotide relative abundances (ρ*XY*) were calculated using the equation ρ*XY* = fXY/fXY where fXY denotes the frequency of dinucleotide XY, and IX and FY denote the frequencies of X and Y, respectively. The difference in genome signature between two sequences is expressed by the genomic dissimilarity (δ*), which is the average absolute dinucleotide of relative abundance difference between two sequences, and were calculated using the equation: δ* = (1/162p*XY (f) - ρ*XY (g)) (multiplied by 1000 for convenience), where the sum extends over all dinucleotides.

Genome-to-genome distances (GGD)

The genome distance was calculated using genome-to-genome distance calculator (GGDC). Distances between a pair of genomes were determined by whole-genome pairwise sequence comparisons using BLAST. For these comparisons, algorithms were used to determine high-scoring segment pairs (HSPs) for inferring intergenomic distances for species delineation. The corresponding distance threshold can be used for species delimitation.
Table 1. Genomic features of the streptococci. G+C content (%): guanine + cytosine content (%). No. of CDs: number of coding DNA sequence. \( Nc \): effective number of codons.

| Organism                     | GenBank accession no. | Genome size (nt) | G+C content (%) | No. of CDS | \( Nc \) |
|------------------------------|-----------------------|------------------|-----------------|-----------|---------|
| S. agalactiae A909           | CP000114              | 2,127,839        | 35              | 1996      | 44.9    |
| S. agalactiae NEM316         | AL732656              | 2,211,485        | 35              | 2094      | 45.2    |
| S. agalactiae 2603VR         | AE009948              | 2,160,267        | 35              | 2124      | 45.1    |
| S. anginosus F0211           | AECT00000000          | 1,993,709        | 38              | 2035      | 50.6    |
| S. bovis ATCC 700338         | AEEL00000000          | 2,050,893        | 37              | 2088      | 44.5    |
| S. downei F0415              | AEKNI00000000         | 2,239,421        | 43              | 2204      | 54.4    |
| S. dysgalactiae subsp. equisimilis GGS-124 | AP010935 | 2,106,340        | 39              | 2094      | 50.3    |
| S. equi subsp. equi 4047     | FM04883               | 2,253,793        | 41              | 2001      | 52.6    |
| S. equi subsp. zooepidemicus | FM04884               | 2,149,868        | 41              | 1869      | 52.4    |
| S. equi subsp. zooepidemicus MGCS10565 | CP001129 | 2,024,171        | 41              | 1893      | 52.3    |
| S. galiliatedicus subsp. galiliatedicus TX20005 | AEEM00000000 | 2,214,091        | 37              | 2218      | 44.5    |
| S. galiliatedicus UCN34      | FN597254              | 2,350,911        | 37              | 2223      | 44.4    |
| S. gordonii str. Challis substr. CH1 | CP000725 | 2,196,662        | 40              | 2051      | 52.4    |
| S. infantis SK1302           | AEDY00000000          | 1,792,252        | 39              | 2102      | 48.9    |
| S. infantarius subsp. infantarius ATCC BAA-102 | ABJK00000000 | 1,925,087        | 37              | 2051      | 44.0    |
| S. mitis B6                  | FN568063              | 2,146,611        | 39              | 2004      | 50.4    |
| S. mitis SK321               | AEDT00000000          | 1,873,702        | 40              | 1757      | 49.8    |
| S. mutans NN2025             | AP010655              | 2,013,587        | 36              | 1895      | 46.4    |
| S. mutans UA159              | AE014133              | 2,030,921        | 36              | 1960      | 46.5    |
| S. oralis ATCC 35037         | AEDW00000000          | 1,884,712        | 41              | 1793      | 51.4    |
| S. parasanguinis ATCC 15912  | ADVN00000000          | 2,124,730        | 41              | 2035      | 52.8    |
| S. parasanguinis F0405       | AEMK00000000          | 2,050,302        | 41              | 1978      | 52.9    |
| S. pneumoniae AP200           | CP002121              | 2,130,580        | 39              | 2216      | 50.3    |
| S. pneumoniae ATCC 700669    | FM211187              | 2,221,315        | 39              | 1990      | 50.0    |
| S. pneumoniae CGSP14         | CP001033              | 2,209,198        | 39              | 2206      | 50.3    |
| S. pneumoniae D39            | CP000410              | 2,046,115        | 39              | 1914      | 49.8    |
| S. pneumoniae G54            | CP000105              | 2,078,953        | 39              | 2114      | 50.0    |
| S. pneumoniae Hungary19A-6   | CP000936              | 2,245,615        | 39              | 2155      | 50.2    |
| S. pneumoniae INV104         | FQ312030              | 2,142,122        | 39              | 1824      | 49.9    |
| S. pneumoniae INV200         | FQ312029              | 2,093,317        | 39              | 1930      | 50.0    |
| S. pneumoniae JJA            | CP000919              | 2,120,234        | 39              | 2123      | 50.2    |
| S. pneumoniae OXC141         | FQ312027              | 2,036,867        | 39              | 1824      | 49.9    |
| S. pneumoniae P1031          | CP000820              | 2,111,882        | 39              | 2073      | 50.1    |
| S. pneumoniae R6             | AE073717              | 2,038,615        | 39              | 2042      | 50.1    |
| S. pneumoniae Taiwan19F-14   | CP000921              | 2,112,148        | 39              | 2044      | 50.1    |
| S. pneumoniae TCH843119A     | CP001993              | 2,088,772        | 39              | 2275      | 50.4    |
| S. pneumoniae TIGR4          | AE05672               | 2,160,842        | 39              | 2105      | 50.0    |
| S. pneumoniae 670-6B         | CP002176              | 2,240,045        | 39              | 2352      | 50.4    |
| S. pneumoniae 70585          | CP000918              | 2,184,682        | 39              | 2202      | 50.1    |
| S. pseudoporphirous SPIN 20026 | AEN5000000000       | 2,111,372        | 36              | 2030      | 48.6    |
| S. pyogenes MGAS315          | AE014074              | 1,900,521        | 38              | 1865      | 49.1    |
| S. pyogenes MGAS2096         | CP000261              | 1,860,355        | 38              | 1898      | 49.4    |
| S. pyogenes MGAS5005         | CP000017              | 1,838,554        | 38              | 1865      | 48.9    |
| S. pyogenes MGAS6180         | CP000056              | 1,897,573        | 38              | 1894      | 48.9    |
| S. pyogenes MGAS8232         | AE009949              | 1,895,017        | 38              | 1839      | 49.0    |
| S. pyogenes MGAS9429         | CP000259              | 1,836,467        | 38              | 1877      | 49.0    |
| S. pyogenes MGAS10270        | CP000260              | 1,928,252        | 38              | 1986      | 49.0    |
| S. pyogenes MGAS10394        | CP000033              | 1,899,877        | 38              | 1886      | 49.2    |
| S. pyogenes MGAS10750        | CP000262              | 1,937,111        | 38              | 1979      | 49.1    |
| S. pyogenes M1 GAS           | AE004092              | 1,852,441        | 38              | 1696      | 48.8    |
| S. pyogenes NZ131            | CP000829              | 1,815,785        | 38              | 1700      | 48.8    |
Results and discussion

In this work we compared complete genomes for 67 streptococci comprising 19 species to address their taxonomic position. A previous study with a small set of streptococci genomes (eight) and species (four), using a combination of several genomic analyses, showed the applicability of this approach in streptococci taxonomy1. Overall our analysis, using a large data set, showed that genomic taxonomy is an accurate approach to clearly define the streptococci species. The taxonomic resolution of the 16S rRNA, AAI, MLSA, GGD and codon usage analysis for streptococci species definition is summarized in Table 2.

General genomic features

The complete genome of the streptococci comprised a single chromosome. The estimated size of the genomes ranged from 1.7 Mb (S. infantis) to 2.3 Mb (S. sanguinis). The number of CDS varied from 1,700 (S. pyogenes) to 2,352 (S. pneumoniae) (Table 1). The average G+C content of streptococci genomes ranged from 35% to 43%. These species presented a variable interspecies genome size and G+C content, indicating heterogeneity within the genus Streptococcus. One of the reasons for this variability could be associated with the frequent occurrence of horizontal gene transfer events4–20.

Phylogenetic reconstructions by 16S rRNA and MLSA

MLSA and 16S rRNA phylogenetic trees showed similar topologies (Figure 1). The MLSA was performed using five instead of the seven genes applied in the pneumococcus multilocus sequence typing (MLST) scheme (http://pneumoniae.mlst.net/). Three genes, aroE, ddl and gki, are from the MLST scheme, and pheS and recA were included in this work. The concatenation of these genes (7741 bp) allowed an accurate delineation of the streptococci species considered here. The nucleotide sequence similarities were much lower for MLSA than 16S rRNA gene. A pairwise comparison of MLSA among the species revealed sequence similarity between 67% and 100%, while the 16S rRNA gene sequence similarities varied from 92% to 100%. At the intraspecies level, the similarity values ranged from 95% to 100% for MLSA, and 99% to 100% for the 16S rRNA gene sequences. The closest species within the Mitis (S. pneumoniae - S. oralis - S. mitis) and Salivarius groups (S. vestibulares - S. salivarius - S. thermophilus) were clearly placed apart from each other by MLSA, while these species had almost identical 16S rRNA gene sequences (≥99% sequence similarity). A previously study showed that recA analysis is a valuable tool for proper identification of pneumococci in routine diagnostics, but limitations on discrimination of other members of the Mitis group were observed19. S. sanguinis ATCC 49296 showed a much closer relationship with S. oralis ATCC 35037T (95% similarity) than to other S. sanguinis strains (77% similarity), suggesting it belongs to the species S. oralis. In addition, S. bovis ATCC 700338 was placed in the S. galactolyticus cluster with 98% MLSA sequence similarity. This work showed that MLSA, using this new combination of five concatenated genes (aroE, ddl, gki, pheS and recA), distinct from the Streptococcus MLST scheme, allowed a proper identification of most streptococci species, even within the VGS group.

Average amino acid identity (AAI)

The percentage of average amino acid identity (AAI) among streptococci species ranges from 68% to 94%, while within species it varies from 95% to 100%. The VGS species S. pneumoniae, S. mitis and S. oralis shared 89–93% AAI. The species S. salivarius, S. thermophilus and S. vestibularis showed a maximum AAI of 93%. S. sanguinis ATCC 49296 and S. oralis ATCC 35037 showed 96% identity and S. bovis ATCC 700338 and S. galactolyticus strains had 98% identity. These findings suggest that strains ATCC 49296 and ATCC 700338 belong to the species S. oralis and S. galactolyticus, respectively. According to our analyses the AAI and MLSA are the most useful genomic features for the elucidation of streptococci taxonomy.

Genome signature

The genomic dissimilarity values among streptococci were between 3 and 127, while the intraspecies values were between 0 and 17. Streptococci within the VGS group, for instance, S. salivarius,
Table 2. Taxonomic resolution of genomic analyses of streptococci species. MLSA: multilocus sequence analysis. AAI: amino acid identity. GGD: genome to genome distance. Nc: effective number of codons.

|                  | 16S rRNA (%) | MLSA (%) | AAI (%) | GGD (%) | Codon usage (Nc) |
|------------------|--------------|----------|---------|---------|------------------|
| **Intraspecies** |              |          |         |         |                  |
| S. pyogenes      | ≥99          | ≥95      | ≥95     | >70     | –                |
| S. agalactiae    | ≥99          | ≥98      | >97     | >70     | 49               |
| S. equi          | 99           | 100      | 98      | >70     | 45               |
| S. suis          | 99           | 98       | >96     | >70     | 52               |
| S. pneumoniae    | 99           | 100      | >97     | >70     | 50               |
| S. thermophilus  | 99           | 100      | >97     | >70     | 47               |
| **Interspecies** |              |          |         |         |                  |
| S. thermophilus-salivarius-vestibularis | 99 | <94 | <93 | <70 | 50–51 |
| S. pneumoniae-mitis-oralis        | >99          | <94      | <93     | <70     |                  |

S. thermophilus and S. vestibularis species, showed dissimilarity values between 5 and 12 and S. pneumoniae, S. mitis and S. oralis species had dissimilarity values between 5 and 14. Thus, there was not a clear differentiation of these closely related species within the VGS group on the basis of the genomic dissimilarity values. This could be due to the extensive recombination and horizontal gene transfer events which occur between closely related streptococci species that share ecological niches.

On the other hand, species within the Pyogenic group had a distinct genomic signature, with values ranging from 13 to 85. However, genome signatures alone have significant limitations when used as phylogenetic markers for differentiating members of the VGS. The exact mechanisms that generate and maintain the genome signatures are complex, but possibly involve differences in species-specific compositional bias, i.e., G+C content, G+C and A+T skews, codon bias, and mutation bias.

Codon usage bias (Nc)

Nc values provide a meaningful measure of the extent of codon preference in a genome, values range between 20 (extremely biased genome where one codon is used per amino acid) and 61 (all synonymous codons are used). Within the set of 67 complete streptococci genomes examined in this study, the Nc ranged from 44.0 to 54.5 (Table 1). For instance, S. pneumoniae - S. oralis - S. mitis species had Nc values of 50, 51 and 50, respectively. The Salivarius group (S. vestibulares - S. salivarius - S. thermophilus), and S. bovis ATCC 700338 - S. galolyticus showed Nc values of 47 and 44.5, respectively. Overall, codon usage bias was very similar among the streptococci species investigated. However, S. sanguinis ATCC 49296 showed a much closer Nc value with the S. oralis ATCC 35037 (51.7 and 51.4, respectively) than other S. sanguinis strains (54.5), which was in agreement with the other analyses used in this study.

Genome distance analysis

The GGD was calculated only for closely related species that were not differentiated by 16S rRNA gene sequence analysis (Figure 1). Based on GGD analysis the species within the Mitis and Salivarius groups were identified as separate species, showing GGD values analogous to the < 70% discriminatory value used for DNA-DNA hybridization. Conversely, S. bovis ATCC 700338 and S. galolyticus were identified as belonging to the same species by GGD.

S. bovis ATCC 700338 (biotype II) and S. galolyticus as well as S. sanguinis ATCC 49296 and S. oralis ATCC 35037T were not separated and, therefore, according to this analysis would be classified as the same species, respectively. It was shown that S. bovis biotype I and II/2 isolates were, in fact, S. galolyticus, and S. sanguinis ATCC 49296 was placed into S. oralis species by GGD analysis. A misidentification of S. sanguinis ATCC 49296 has already been shown by means of biochemical and serological properties by Narikawa and colleagues.

Another interesting result is that the S. parasanguinis ATCC 15912 and F0405 strains were found to be at the upper limits for definition as members of the same species based on different genomic analyses. For instance, they shared 95% AAI, 94% identity by MLSA, a value of 17 on the basis of genomic signature and < 70% similarity in GGD. Therefore, based on these genomic
Figure 1. Neighbor-joining tree based on 16S rRNA gene sequences and MLSA concatenated sequences of *Streptococcus*. The numbers at the nodes indicate the values of bootstrap statistics after 2000 replications, and values below 50% are not shown. Bars, 0.005% and 0.02% estimated sequence divergence.
markers, these *S. parasaurophilus* strains could, in fact, be separate species. This data reflects the complexity of bacterial species delineation, since these organisms are all under a constant evolutionary process.

## Conclusion

The delineation of closely related streptococci species was evident in this genomic study. Different methods produced different levels of taxonomic resolution. The methods with the higher resolution for species identification were MLSA and AAI, while closely related species had similar *Nc* values and genomic signatures. Based on the genomic analyses, a *Streptococcus* species can be defined as a group of strains that shares ≥ 95% identity in MLSA and AAI, and > 70% identity in GGD. This definition may be useful to advance the taxonomy of *Streptococcus*. This approach allows an advanced understanding of bacterial diversity and identification.

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### Author contributions

CCT and VEE carried out the computational and genomic analyses and analyzed the results. All authors (ACPV, CCT, ELF, MAM and VEE) participated in discussing and writing the manuscript. All authors have agreed to the final contents of the article.

## Competing interests

No relevant competing interests were disclosed.

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Referee Responses for Version 1

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Approved: 03 April 2013

Referee Report: 03 April 2013
The article is well written with an appropriate title and abstract. The methods are adequate for the aims of the study, but I would suggest that including the Average Nucleotide Identity (ANI) analysis as suggested by Rosello-Mora et al. 2006, would certainly improve the manuscript. The online analysis can be found here http://www.imedea.uib.es/jspecies/index.html. The conclusions are adequate and the data sufficient to replicate all the analyses. The data are openly accessible at GenBank.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Tomoo Sawabe
Laboratory of Microbiology, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Japan

Approved: 03 April 2013

Referee Report: 03 April 2013
- Title and abstract are good enough to attract readers in the scientific community.
- Genome based multi-gene sequence comparison is one of the promising tools to analyse bacterial populations. To achieve the analysis for Streptococcus, the authors carefully designed massive data genome analysis. The results are strong enough and supported by the results of the analysis.
- The conclusion is clear that authors proposed a threshold value on the basis of genome-based MLSA in Streptococcus bacteria.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.