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Phylogenetic analysis supports horizontal gene transfer of L-amino acid oxidase gene in *Streptococcus oligofermentans*

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Abstract

Phylogenetic analysis of 10 amino acid sequences from 19 *Streptococcus* species showed that *S. oligofermentans* clustered within the mitis group. However, the L-amino acid oxidase (LAO) of *S. oligofermentans* showed a different clustering pattern from the other proteins analyzed implicating horizontal gene transfer (HGT) in the origin of the *S. oligofermentans* LAO gene. LAO of *S. oligofermentans* is known to confer ability to compete with other oral cavity bacteria, most notably *S. mutans*; therefore, the HGT event may have been important in extending the ecological niche occupied by this species, consistent with those of other studies suggesting that HGT can play a key role in enabling bacterial species to occupy new ecological niches.

Keywords

horizontal gene transfer; L-amino acid oxidase; *Streptococcus oligofermentans*

1. Introduction

The genus *Streptococcus* contains several species of economic and medical importance, including species that are commensals or pathogens of man and domestic animals (Delorme et al. 2010; Glazunova et al., 2006; Matta et al., 2009). Among the most important impacts of *Streptococcus* on human health is their role in dental caries, which is one of the most prevalent bacterial diseases affecting human populations (Petersen 2003; Petersen et al. 2005). The primary source of cariogenesis can be isolated to one microbial species, *S. mutans*, which was first described by Fitzgerald and Keyes (1960) as the etiological agent of cariogenesis. *S. mutans* is only one of many species among the oral flora which produce cariogenesis through acidogenesis. However, *S. mutans* displays the unique ability to bond to the tooth surface using a mechanism involving the protein coded by *spaP* (Adjic et al., 2002). The ability to bond to an uncolonized tooth surface sets *S. mutans* apart as a gateway species. After *S. mutans* colonization more fastidious bacteria colonize through interspecies glucan interactions and further the demineralization of the tooth structure through acid production.

S. oligofermentans is a relatively novel species first described by Tong et al. in 2003. The species' importance revolves around its ability to inhibit *S. mutans* in the oral environment.

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Studies in the Chinese population have confirmed that *S. oligofermentans* in addition to being non- cariogenic, is also found on tooth surfaces free of *S. mutans*. *S. oligofermentans*' inhibitory properties towards *S. mutans* have been mapped to the *lox* gene, encoding L-amino acid oxidase gene (LAAO; Tong et al. 2007, 2008). A knockout of the *lox* gene in *S. oligofermentans* lost inhibitory properties towards *S. mutans* (Tong et al., 2008). This inhibitory capacity, along with the lowered tooth surface-binding ability of *S. oligofermentans* and lowered production of acids which cause hydroxyapatite demineralization in the teeth, suggest that *S. oligofermentans* may be useful in prophylactic measures against dental caries (Zhang et al., 2010).

The genus *Streptococcus* has been previously categorized based on phylogenies constructed using 16S rRNA into six groups: pyogenic, anginosus, mitis, salivarius, bovis, and mutans groups (Kawamura et al., 1995). Though widely used for bacterial phylogeny, 16s rRNA sequences are not always useful in the study of closely related species because of high sequence identity due to functional constraint. Thus, protein-coding genes have also been used, as illustrated by a phylogenetic analysis of the mitis group based on partial manganese-dependent superoxide dismutase gene (*sodA*) sequences (Kawamura et al., 1999).

In the present study, we reconstructed phylogenetic relationships of selected *Streptococcus* isolates based on the amino acid sequences encoded by *sodA* and 9 other genes, including the gene encoding LAAO. Our goals were two fold: (1) to examine the evolutionary relationship between *S. oligofermentans* and the major previously identified groups of *Streptococcus*; and (2) to compare the phylogenies reconstructed using different genes in order to test for evidence of recombinational events in their evolutionary histories, as indicated by phylogenetic inconsistencies between genes.

2. Materials and Methods

2.1 Amino acid sequences

S. oligofermentans amino acid sequences from the Genbank database were used in a BLAST homology search in order to identify homologs in other *Streptococcus* species (Supplementary Table S1). In the analysis we used all amino acid sequences available in the database from the following taxa: *S. oligofermentans* and 18 other *Streptococcus* isolates (*S. species 2* 136FAA, *S. thermophilus*, *S. suis*, *S. sanguinis*, *S. salivarius*, *S. pyogenes*, *S. pneumoniae*, *S. parasanguinis*, *S. oralis*, *S. oral species*, *S. mutans*, *S. mitis*, *S. M143*, *S. gordonii*, *S. gallolyticus*, *S. dysgalactiae*, *S. bovis*, and *S. agalactiae*) and an outgroup within the family Streptococcaceae, *Lactococcus lactis*. Because multiple strains within species were available only in a few cases and because in *Streptococcus* within-species sequence differences are generally much lower than between-species differences (Pombert et al. 2009), for each protein we chose only a single sequence from each species, with preference for sequences from completely sequenced genomes.

The genes analyzed were the following: *PheS*, encoding phenylalanyl-tRNA synthase alpha subunit; *atpA* encoding ATP synthase F1 sector subunit alpha; *LAAO* encoding L-amino oxidase; *rpoB* encoding the DNA-directed RNA polymerase beta chain; *sodA* encoding superoxide dismutase; *groEL* encoding the chaperonin groE; *gyrB* encoding DNA gyrase subunit B; *recN* encoding DNA repair protein recN; *atpD* encoding ATP synthase F1 sector subunit beta; and *EF-TU* encoding elongation factor TU (Supplementary Table S1). These genes are widely scattered throughout Streptococcus genomes. For example, in the sequenced genome of *S. gordonii* (NC_009785), the region including the 10 genes spans 1.2 megabases out of the 2.2 megabases in the genome.

2.2 Sequence alignment and phylogenetic reconstruction

Amino acid sequences were aligned using the CLUSTAL algorithm in MEGA 5 (Tamura et al. 2011). A Bayesian phylogenetic analysis of the concatenated alignment of all 10 amino acid sequences was conducted using the Mr. Bayes software (Huelsenbeck and Ronquist 2001) and the amino acid WAG + gamma model (Whelan and Goldman 2001), with four rate categories. The WAG + gamma model was chosen by the Mr. Bayes software as the most appropriate model for these data. Four chains were run for 1,700,000 generations with a sample taken every 100 generations producing a total of 34,002 trees separated equally into two files of which Bayesian posterior probabilities were inferred from 25,502 trees (12,751 per file). The average standard deviation of split frequencies was 0.005166.

We likewise conducted maximum Likelihood (ML) phylogenetic analyses based on the WAG + gamma model with eight discrete rate categories, using the MEGA 5 software (Tamura et al. 2011). ML analyses were conducted for concatenated sequences; separately for each of the 10 proteins; and separately for the concatenated set, leaving out each protein in turn. Confidence of branching patterns within the ML phylogeny was assessed by bootstrapping (Felsenstein 1985); 1000 bootstrap samples were used. Most probable ancestral sequences were reconstructed by the ML method in MEGA 5.

2.3. Other statistical methods

We used the RDP3 program (Martin et al. 2010) to test for intragenic recombination. As a test for recombinational events involving entire genes, matrices of pairwise JTT + gamma distances amino acid distances were computed in MEGA 5, using shape parameters estimated by the ML method in MEGA 5 (Tamura et al. 2011). For each protein, the pairwise JTT+ gamma distances were compared with that of the concatenated set of the nine remaining proteins. Plots of the pairwise amino acid distances for a given protein vs. those for the concatenated set of the 9 other proteins were used to identify outliers possibly indicative of recombination.

A randomization test was used to test for significance of outliers. This randomization test was based on the regression of the distance in the protein of interest (Y) vs. that in the 9 other proteins (X) and the computation of standardized residuals from that regression. Regression through the origin was used because it is expected that both X and Y would be zero immediately after divergence of two lineages. We created 1000 simulated samples by drawing randomly (with replacement) from the observed X and Y values. Each simulated sample contained as many bivariate data points (n) as the original sample. We computed the linear regression for each simulated sample and the absolute value of each standardized residual for each simulated sample.

To test whether a set of m standardized residuals from the original regression were significantly greater than expected, we compared the mean of the absolute value of those m standardized residuals with the mean of the absolute values of the remaining $n - m$ standardized residuals. We used as a test statistic the absolute difference (D) between the mean of the absolute values of the m standardized residuals in the set of interest and the mean of the absolute values of the remaining $n - m$ standardized residuals. The value of D in the actual data was compared with D values computed from groups of size m drawn at random from the simulated samples. For a two-tailed test, D in the actual data was considered significant at the α level if it was greater than the maximum D computed in 100(1- α)% of the 1000 simulated samples.

3. Results

3.1. Phylogenetic analyses

The maximum likelihood phylogeny based on the concatenated set of all 10 amino acid sequences showed a cluster with 100% bootstrap support containing *S. M143*, *S. oralis*, *S. mitis*, *S. pneumoniae*, *S. oral species*, and *S. oligofermentans* (Figure 1). With 77% bootstrap support, the latter group clustered with *S. sanguinis*, *S. species 2 1 36FAA*, and *S. gordonii* (Figure 1). In addition, *S. parasanguinis* clustered with the above groups, and this 10-taxon cluster received 99% bootstrap support (Figure 1). The latter cluster seemed to correspond to the mitis group as previously defined (Kawamura et al. 1995). The phylogeny constructed based on Bayesian methods showed identical clustering for the above-mentioned 10 mitis-group species (Supplementary Figure S1).

When ML phylogenies were reconstructed for each of the 10 individual amino acid sequences, 6 of 10 of the phylogenies clustered *S. M143*, *S. oralis*, *S. mitis*, *S. pneumoniae*, *S. oral species*, and *S. oligofermentans* (Figure 2 and Supplementary Figures S2–S10), as in the phylogeny based on the concatenated sequences (Figure 1). Likewise, 8 of 10 ML phylogenies clustered *S. sanguinis*, *S. species 2 1 36FAA*, and *S. gordonii* together (Figure 2 and Supplementary Figures S2–S10), as in the phylogeny based on the concatenated sequences. On the other hand, the above-mentioned 10-taxon mitis group cluster including the latter groups along with *S. parasanguinis* was seen in just 4 of 10 ML phylogenies (Figure 2 and Supplementary Figures S2–S10).

In the ML tree of LAAO sequences, the 10 mitis group taxa clustered together with 68% bootstrapping (Figure 2), but a significant deviation from the tree based on concatenated sequences was seen in the position of *S. oligofermentans* within that group (Figures 1–2). In the LAAO tree, *S. oligofermentans* clustered with *S. sanguinis*, *S. species 2 1 36FAA*, and *S. gordonii* with 96% bootstrap support (Figure 2), whereas in the tree based on the concatenated sequences, *S. oligofermentans* clustered with *S. M143*, *S. oralis*, *S. mitis*, *S. pneumoniae*, and *S. oral species* with 100% bootstrap support (Figure 1). In a tree based on all concatenated sequences except LAAO (Figure 3), the position of *S. oligofermentans* remained the same as that seen in the tree based on all concatenated sequences, again with 100% bootstrap support (Figure 3).

3.2. Tests for recombination

One possible explanation for the different phylogenetic position of LAAO of *S. oligofermentans* in comparison to the other proteins is an intragenic recombination event covering just part of the *LAAO* gene. We tested for intragenic recombination in the *LAAO* gene of *S. oligofermentans* using RDP3. Because tests for recombination work best with relatively closely related sequences, we used in these analyses the DNA sequences of the *LAAO* gene from the 10 mitis-group taxa: *S. oligofermentans*, *S. oral species*, *S. M143*, *S. oralis*, *S. pneumoniae*, *S. mitis*, *S. species 2 1 36FAA*, *S. gordonii*, *S. sanguinis*, and *S. parasanguinis*. These taxa include the two major clades involved in the apparent recombination (Figures 2 and 3). In the *LAAO* genes of these taxa, the RDP, GeneConv, and MaxChi algorithms in RDP3 failed to detect any significant evidence of intragenic recombination.

The ML method was used to reconstruct the most probable ancestral amino acid sequences for the phylogeny of LAAO (Figure 2). A total of 15 amino acid replacements were predicted to have taken place on the branch leading to the common ancestor of LAAO from *S. oligofermentans*, *S. sanguinis*, *S. gordonii*, and *S. species 2 1 36FAA* (Figure 3). These 15 amino acid replacements occurred at the following positions, numbered following *S. oligofermentans* LAAO: 32, 57, 145, 147, 156, 280, 283, 289, 296, 305, 306, 307, 311, 314,

and 315. (For alignment of LAAO sequences used in this study, see Figure S11). The fact that these amino acid replacements were scattered along the sequence of the LAAO protein provides additional evidence against the hypothesis of an intragenic recombination.

Using the same 10 mitis-group taxa, we plotted amino acid distances for LAAO vs. those for the concatenated data set excluding LAAO (Figure 4). There were some striking outliers indicative of unusual patterns of amino acid distance (Figure 4). The comparisons between, on the one hand, *S. oligofermentans* and, on the other hand, *S. M143*, *S. oralis*, *S. mitis*, *S. pneumoniae*, and *S. oral species* provided strongly positive outliers (Figure 4). By contrast, the comparisons between, on the one hand, *S. oligofermentans* and, on the other hand, *S. species 2 1 36FAA*, *S. gordonii*, and *S. sanguinis* provided strongly negative outliers (Figure 4). These 8 outliers, the mean absolute value of the standardized residual from a linear regression line through the origin was 2.054 ± 0.136 SE, while that for the remaining points was 0.377 ± 0.054 . The difference between the means was highly significant ($P < 0.001$; randomization test). This result was consistent with that of the phylogenetic analysis (Figure 2), in showing an unusual pattern in the LAAO of *S. oligofermentans*.

4. Discussion

Previous phylogenetic analyses based on 16S rRNA and the *sodA* gene have supported monophyly of the mitis group of *Streptococcus* (Kawamura et al. 1995, 1999), but they have not clearly resolved relationships within the mitis group. The present analysis, based on 10 amino acid sequences, defined three well-supported subgroups within the mitis group: (1) a group including *S. M143*, *S. oralis*, *S. mitis*, *S. pneumoniae*, *S. oral species*, and *S. oligofermentans*; (2) a group including *S. sanguinis*, *S. species 2 1 36FAA*, and *S. gordonii*; and (3) a basal group, of which the only representative in the present study was *S. parasanguinis*. The present analysis thus confirmed the position of *S. oligofermentans* in the mitis group (Tong et al. 2003) and supported a close relationship between *S. oligofermentans* and a specific subgroup of taxa within the mitis group.

Both homologous recombination and non-homologous recombination are thought to play important roles in bacterial evolution, by introducing new allelic variants within a genome (Daubin et al. 2003; Hughes and Langley 2007; Lerat et al. 2005; van Passel et al. 2008). Evidence for recombinational or “horizontal gene transfer” (HGT) events can be derived by a variety of methods, but one of the most reliable approaches involves the application of phylogenetic analysis separately to multiple genomic regions (Daubin et al. 2003). A gene that shows a markedly different pattern of relationship from others in the genome is likely to have been derived by recombination with a distantly related genome. Such an anomaly was observed in the present analysis in the case of LAAO. The results suggested that *S. oligofermentans* received the gene encoding LAAO by HGT from a source closely related to *S. sanguinis*, *S. gordonii*, and *S. species 2 1 36FAA*, whereas other proteins of *S. oligofermentans* show a closer relationship to *S. M143*, *S. oralis*, *S. mitis*, *S. pneumoniae*, and *S. oral species*.

Because of the importance of LAAO in the ability of *S. oligofermentans* to compete with other oral cavity bacteria, most notably *S. mutans*, it is a plausible hypothesis that the HGT event was important in extending the ecological niche occupied by this species. This hypothesis might be tested experimentally by replacing the LAAO gene in *S. oligofermentans* with one from *S. oralis* or a similar species and observing the effects of such a substitution on the ability of *S. oligofermentans* to compete with *S. mutans*. Our results are consistent with those of other studies suggesting that HGT can play a key role in the origin of bacterial adaptations (Marri et al. 2006, 2007; Richards et al. 2011; Scholten et al. 2007; Sumby et al. 2005; Whittam and Bumbaugh 2002). The hypothesized HGT event

in the case of the LAAO gene of *S. oligofermentans* involved gene transfer between the two major subclades of the mitis group. When a complete genomic sequences of *S. oligofermentans* and other taxa in the mitis group become available, it will be possible to understand further the relationships within this group and to test whether the HGT of LAAO was a unique event or whether other genes were involved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research highlights

- Phylogenetic analysis showed relationship of *Streptococcus oligofermentans* to other species of *Streptococcus*
- L-amino acid oxidase (LAAO) of *S. oligofermentans* confers ability to compete in oral environment
- L-amino acid oxidase (LAAO) of *S. oligofermentans* was obtained by horizontal transfer from other *Streptococcus* group

Figure 1.

ML tree based on 10 concatenated amino acid sequences (2561 aligned amino acid sites). Numbers on the branches represent percentage of bootstrap samples supporting the branch; only values ≥ 50% are shown.

Figure 2. ML tree of LAAO sequences (389 aligned amino acid sites). Numbers on the branches represent percentage of bootstrap samples supporting the branch; only values ≥ 50% are shown.

Figure 3.

ML tree based on 9 concatenated amino acid sequences (excluding LAAO; 2172 aligned amino acid sites). Numbers on the branches represent percentage of bootstrap samples supporting the branch; only values $\geq 50\%$ are shown.

Figure 4.

Plot of pairwise amino acid distance (JTT + gamma) in LAAO vs. that in 9 other proteins for the 10 mitis-group taxa. *Solid circles* indicate comparisons of *S. oligofermentans* vs. *S. M143*, *S. oralis*, *S. mitis*, *S. pneumoniae*, and *S. oral species*. *Solid diamonds* indicate comparisons of *S. oligofermentans* vs. *S. species 2 1 36FAA*, *S. gordonii*, and *S. sanguinis*. All other comparisons are indicated by open circles. The line is the linear regression line through the origin, $Y = 1.98X$. The shape parameter for the gamma distribution was estimated by the ML procedure in MEGA 5; $a = 0.286$ in the case of LAAO and $a = 0.143$ in the case of the other proteins.