Abiotrophia elegans Strains Comprise 8% of the Nutritionally Variant Streptococci Isolated from the Human Mouth

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Ninety-one isolates of nutritionally variant streptococci (NVS) that were previously isolated from the human mouth were regarded as consisting of 7 Streptococcus defectivus isolates, 78 Streptococcus adjacens isolates, and 6 Gemella morbillorum isolates. However, recent references to the taxonomic reclassification of NVS, from S. defectivus to Abiotrophia defectiva and from S. adjacens to Abiotrophia adiacens, and the newly introduced species Abiotrophia elegans as a third Abiotrophia species, emphasize the need for genetic analyses for identification of NVS. When PCR-restriction fragment length polymorphism (RFLP) and phylogenetic distances were examined based on 16S rRNA gene sequences, the results indicated that 7 of the 91 NVS isolates were closely related to A. elegans. These seven isolates consisted of four isolates previously identified as G. morbillorum and three isolates previously identified as S. adjacens. Two isolates previously identified as G. morbillorum were related to A. adiacens. In biochemical tests, A. elegans and the seven isolates related to it possessed arginine dihydrolase (ADH) activity but the other Abiotrophia species did not. As a result, A. elegans strains comprised 8% of the 91 NVS isolates. Our findings suggest that A. elegans, A. adiacens, and A. defectiva exist in the human mouth in proportions of about 1:11:1 and that A. elegans can be genetically distinguished from the other two Abiotrophia species by PCR-RFLP analysis of 16S rRNA gene sequences and can be biochemically distinguished by ADH activity.

NUTRITIONALLY VARIANT STREPTOCOCCI (NVS) can be seen as satellite colonies around other microorganisms and require cysteine or vitamin B₆ for growth in complex medium (3, 13). Although such streptococci are responsible for a variety of infectious diseases (13), they have been isolated not only from flora associated with disease but also from normal flora in the form of symbiotic streptococci (5). In particular, occurrence of NVS in the human mouth is typical (6, 9, 10).

With respect to taxonomy, in 1989, Bouvet et al. identified Streptococcus defectivus and Streptococcus adjacens as new species of NVS based on their different biochemical characteristics and DNA homology (2). Then, in 1995, Kawamura et al. proposed a new genus, Abiotrophia, based on the phylogenetic distances of 16S rRNA gene sequences and named two species, Abiotrophia defectiva and Abiotrophia adiacens, based on the species names S. defectivus and S. adjacens, respectively (7). In 1998, Roggenkamp et al. specified the 16S rRNA gene sequences, biochemical characteristics, and growth characteristics for a third Abiotrophia species, Abiotrophia elegans (12). They also showed the differentiation among these three species by PCR amplification with various primers which had sequences found in 16S rRNA genes (11). Thus, at this moment, NVS are regarded as comprising three Abiotrophia species: A. defectiva, A. adiacens, and A. elegans.

In 1996, we reported 91 NVS isolates from the human mouth (6). All of them presented bacteriolytic activity and pink chromopores and required additional vitamin B₆ for their growth. The results of identification with a Rapid ID32 STREP kit showed that these 91 NVS isolates consisted of 7 S. defectivus isolates, 78 S. adjacens isolates, including NMP3, S94-2, and S1052-1, and 6 Gemella morbillorum isolates. Despite this identification, the six isolates previously identified as G. morbillorum, i.e., C9-2, HHC5, HKT1-1, S43-1, TKT2, and YTM1, present an as yet unsolved problem, since G. morbillorum is able to grow without additional vitamin B₆ and presents neither bacteriolytic activity nor chromophores (3).

For identification of NVS, recent studies on Abiotrophia species have emphasized the need for genetic analyses, particularly for a PCR assay based on 16S rRNA gene sequences. In order to solve the problem presented above, we reexamined the 91 isolates of NVS by such genetic analyses. The observed characteristics and resulting reidentifications are presented here.

MATERIALS AND METHODS

Strains and culture conditions. The 91 NVS, which included C9-2, HHC5, YTM1, TKT2, and S43-1, were previously isolated from a healthy human mouth in our laboratory (6). A. defectiva ATCC 49176T, A. adiacens ATCC 49175T, and G. morbillorum ATCC 27824T were purchased from the American Type Culture Collection (Manassas, Va.). A. elegans DSM 11693T was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Bacteria, with the exception of A. elegans DSM 11693T, were grown in Todd-Hewitt broth (ThB; BBL, Becton Dickinson and Company, Cockeysville, Md.) containing 0.001% pyridoxal hydrochloride. A. elegans DSM 11693T was grown in THB containing 5% horse serum and 0.01% l-cysteine hydrochloride.

DNA extraction for PCR. Cells in 1 ml of culture were collected, suspended in 0.2 ml of lysis buffer, and boiled for 3 min based on the method of Watanabe and Frommel (16). After centrifugation, DNA-containing supernatant was obtained.

Primer sequences. A pair of primers corresponding to Escherichia coli 16S rRNA gene positions 8 to 27 (5′-AGGTTTGATCCTAGGTCA-3′) (17) and 1405 to 1391 (5′-ACGGCCGTTGTTGAC-3′) (8) was obtained from Shiga, Japan) was used. A mixture of DNA extract (0.5 μl), 0.2 μM primers, and Taq DNA polymerase (Premix Taq; Takara Shuzo Co., Ltd., Shiga, Japan) in a 50-μl volume was incubated for 30 cycles of 94°C for 1 min and 64°C for 1 min and for an extension at 64°C for 10 min with a Zymoreactor II thermal cycler (Atto Corporation, Tokyo, Japan).

PCR-restriction fragment length polymorphism (RFLP) analysis. The PCR product (20 μl) was digested first with KpnI in low-salt buffer, then with HindIII in medium-salt buffer, and finally with PstI in high-salt buffer at 37°C for 1 h for each digestion (final volume, 30 μl). Five units of each restriction enzyme (Nippon Gene, Osaka, Japan) was used. The triple enzyme digest (12 μl) was analyzed in a 2.5% ethidium bromide-stained agarose gel.

16S rRNA gene sequence of HHC5. The PCR product was cloned into a pCR2.1 vector (TA cloning kit; Invitrogen Corporation, Carlsbad, Calif.) and cut with EcoRI fragments. The single-stranded DNAs were obtained by subcloning into M13mp18 and sequenced by using a Thermo Sequenase premix

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cycle sequencing kit (Amersham) with an automatic sequencer (model Hitachi Ltd., SQ-5500; Tokyo, Japan). The HHC5 and the other sequences derived from data deposited with the DNA Data Bank of Japan (DDBJ, Mishima, Japan) were analyzed, and the results were used to construct a phylogenetic tree by means of a DDBJ Super Computer (Fujitsu VPP500) and the program Clustalw (supplied by EMBL).

DNA-DNA hybridization. Chromosomal DNA was extracted as described previously without treatment with achromopeptidase (15). The DNA (10 µg) was loaded onto a Hybond-N+ membrane (Amersham Pharmacia Biotech) and hybridized with [α-32P]dCTP (NEN, Boston, Mass.)-labeled DNA in 25 mM phosphate buffer (pH 6.5) containing 30% formamide, 3× SSC (3× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt’s solution, and salmon sperm DNA (0.2 mg/ml) at 42°C for 24 h. The membrane was washed with 2× SSC–0.1% SDS at 52°C (15 min) and then with 0.2× SSC–0.1% SDS at room temperature (10 min). Radioactivity of the hybridized DNA was quantified with a BAS 1000 bioimaging analyzer (Fuji Photo Film Co., Tokyo, Japan). Triplicate tests were run for each assay, and the data were normalized with the value for the homologous DNA-DNA hybridization taken as 100%.

Biochemical characterization. Bacteriolytic activity on Micrococcus luteus was tested as described previously (10). To examine an essential growth factor, cells were cultured in brain heart infusion broth (BHIB), BHIB containing 0.01% pyridoxal hydrochloride (vitamin B6), or BHIB containing 0.01% L-cysteine (12). The activities of 32 enzymes were tested by using a Rapid ID32 STREP kit (BioMérieux S.A., Marcy l’Etoile, France).

RESULTS

Comparison of PCR-RFLP patterns among NVS, Abiotrophia species, and G. morbillorum. PCR-RFLP analysis of the 16S rRNA gene (Fig. 1) showed that A. defectiva produced one PCR product of 1,400 bp which was notdigested by any of the three enzymes. However, A. adiacens and G. morbillorum each produced PCR products which were digested into three fragments of 650, 550, and 210 bp and of 550, 490, and 370 bp, respectively. On the other hand, the new species, A. elegans, produced a PCR product which was digested into two fragments of 860 and 550 bp. Surprisingly, 7 of the 91 clinical isolates produced PCR products which were digested into two fragments of the same sizes as those produced by A. elegans. These results suggested that the seven isolates might be A. elegans.

This finding was unexpected, since our isolates can grow in the presence of vitamin B6 without l-cysteine in the medium, like A. defectiva and A. adiacens, while the original A. elegans isolate requires specific addition of l-cysteine to the medium for growth (12).

Of the remainder of our isolates, 77 presented three digested fragments similar in length to those produced by A. adiacens and 7 presented nondigested fragments similar to those produced by A. defectiva (data not shown). As a result, the PCR-RFLP analysis of the 16S rRNA genes readily allocated all 91 NVS isolates among the three Abiotrophia species: 7 isolates as A. defectiva, 77 isolates as A. adiacens, and 7 isolates as A. elegans.

The seven isolates of A. defectiva correctly corresponded to the seven isolates previously identified as S. defectivus, while the seven new A. elegans isolates consisted of three previously identified as S. adiacens and four previously identified as G. morbillorum. Thus, the 77 isolates of A. adiacens consisted of 75 previously identified as S. adiacens and 2 previously identified as G. morbillorum.

16S rRNA gene sequence of the isolate HHC5. The HHC5 sequence, which was typical of the seven isolates described above, was 1,407 bp in length and included the forward and reverse primers in the 5’ and 3’ directions, respectively. The result of a multiple alignment analysis showed that the HHC5 sequence resembled A. elegans more than it did the other two Abiotrophia species and resembled G. morbillorum less than it did the Abiotrophia species. The homologies between HHC5 and each of A. elegans, A. adiacens, A. defectiva, and G. morbillorum were 99, 97, 93, and 86%, respectively.

An unrooted phylogenetic tree (Fig. 2) clearly revealed that HHC5 was highly homologous to A. elegans but considerably dissimilar to G. morbillorum, despite the previous identification of HHC5 as G. morbillorum (6).

The findings that the phylogenetic distance between A. elegans and HHC5 was the shortest observed and that the homology between the two sequences was significant (99% identity) strongly supported the notion that the seven isolates (Fig. 1, lanes 6 to 12) are A. elegans, despite the different factors required for their growth.

Restriction enzyme sites in 16S rRNA gene. Agarose gel electrophoretic analyses of the restriction enzyme digests and the data for the DNA sequences revealed that in the 16S rRNA gene A. defectiva did not possess HinIII, PstI, or KpnI sites, A. adiacens possessed HinIII and PstI sites located at positions 214 and 862, respectively, and A. elegans possessed a PstI site located at position 863. Only G. morbillorum possessed...
Table 2. Critical biochemical characteristics specific to *A. elegans* and seven related isolates

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<tr>
<th>Bacterial strain or isolate (no. of isolates)</th>
<th>Characteristic (no. of isolates)</th>
<th>Lytic enzyme production</th>
<th>Vitamin B&lt;sub&gt;6&lt;/sub&gt;</th>
<th>L-Cysteine&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Activity</th>
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<td>ADH</td>
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<td><em>A. defectiva</em> ATCC 49176&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>Clinical isolates identified as <em>A. defectiva</em> (7)</td>
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<td><em>A. adiacens</em> ATCC 49175&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>Clinical isolates identified as <em>A. adiacens</em> (77)</td>
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<td><em>A. elegans</em> DSM 11693&lt;sup&gt;T&lt;/sup&gt;</td>
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<td><em>G. morbillorum</em> ATCC 27824&lt;sup&gt;T&lt;/sup&gt;</td>
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<sup>a</sup> Ability to grow in vitamin B<sub>6</sub>-supplemented medium without l-cysteine.
<sup>b</sup> Ability to grow in l-cysteine-supplemented medium without vitamin B<sub>6</sub>.
<sup>c</sup> *G. morbillorum* required neither vitamin B<sub>6</sub> nor l-cysteine for growth.

**DISCUSSION**

NVS, initially regarded as consisting of *S. adjacens* and *S. defectivus*, are now classified into *A. elegans*, *A. adiacens*, and *A. defectiva* by genetic analyses (7, 9, 11, 12). We reexamined genetically the identification of 91 isolates of NVS. As shown in Fig. 1, the 16S rRNA genes of seven of those isolates presented the same PCR-RFLP pattern as *A. elegans* DSM 11693<sup>T</sup>. Sequence analysis of HHC5, the most typical of the seven isolates, also showed that HHC5 was most related to *A. elegans* (99% identity) and had the shortest phylogenetic distance from it (Fig. 2). These results strongly indicated that the seven isolates were *A. elegans*. Further results of DNA-DNA hybridization (Table 1) (14) led us to conclude that the seven isolates were *A. elegans*.

Although HHC5 was previously identified as *G. morbillorum* (6), the present genetic analyses clearly showed that this isolate was unrelated to *G. morbillorum*. This finding is reasonable since the fundamental characteristics of HHC5 are different from those of *G. morbillorum* (Table 2).

The seven isolates genetically related to *A. elegans* consisted of four isolates previously identified as *G. morbillorum* and three isolates previously identified as *S. adjacens*. In addition, two isolates previously identified as *G. morbillorum* were reidentified as *A. adiacens* (data not shown), but the seven *A. defectiva* isolates completely corresponded with the seven isolates previously identified as *S. defectivus*. These results suggest that the results of genetic analysis and biochemical identification correspond exactly for *A. defectiva* but not for *A. elegans* and *A. adiacens*. They also indicate that *A. elegans* and *A. adiacens* isolates are sometimes identified as *G. morbillorum* isolates based on a biochemical identification system, e.g., the Rapid ID32 STREP kit.

In this experiment, DSM 11693 (12) was used as a type strain of *A. elegans*; however, only DSM 11693<sup>T</sup> was unable to grow in the vitamin B<sub>6</sub>-containing medium without added l-cysteine (Table 2). We are very interested in the requirement for l-cysteine of other *A. elegans* strains (11).

Roggenkamp et al. demonstrated that the polypeptide profiles differed among *A. defectiva*, *A. adiacens*, and *A. elegans* (12). Further, Collins et al. showed a similarity dendrogram based on whole-cell protein patterns which clearly indicated that *A. defectiva*, *A. adiacens*, *A. elegans*, and *G. morbillorum* belong to independent clusters (4). The protein profiles resulting from the present SDS-polyacrylamide gel electrophoresis analysis also suggested that HHC5 more closely resembled...
A. elegans than it did either A. defectiva or A. adiacens (data not shown).

Beighton et al. reported enzymatic activities differentiating S. defectivus from S. adiacens (1), but A. elegans-specific enzyme activities have not yet been described. When we reexamined the biochemical characteristics, ADH activity was demonstrated as the only characteristic specific to A. elegans DSM 11693 and the seven related isolates (Table 2). URE activity was also a critical characteristic in identifying some isolates, as described in the Results section. However, the specificity of ADH and URE activities would have to apply to a large number of A. elegans isolates before we could infer that these characteristics distinguish A. elegans from the other Abiotrophia species.

Our reassignment of seven isolates to the species A. elegans brings the percentage of A. elegans to all isolates of NVS in the human mouth to 7.7%. This proportion is the same as that for A. defectiva. Ohara-Nemoto et al. did not estimate the proportion of A. elegans isolates, but they noted colonization frequencies of 11.8% for A. defectiva and 87.1% for A. adiacens for 92 isolates from normal flora found in the human oral cavity (9). Of our Abiotrophia isolates 84.6% were A. adiacens, which seems to be a reasonable amount. We plan to study the frequencies of A. elegans relative to A. adiacens and A. defectiva in other isolates from normal human flora.

REFERENCES