

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 chromophores and required additional vitamin B₆ in the medium 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, S943-2, 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, S43-1, NMP3, S943-2, and S1052-1, were previously isolated from a healthy human mouth in our laboratory (6). *A. defectiva* ATCC 49176^T, *A. adiacens* ATCC 49175^T, and *G. morbillorum* ATCC 27824^T were purchased from the American Type Culture Collection (Manassas, Va.). *A. elegans* DSM 11693^T was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Bacteria, with the exception of *A. elegans* DSM 11693^T, were grown in Todd-Hewitt broth (THB; BBL Becton Dickinson and Company, Cockeysville, Md.) containing 0.001% pyridoxal hydrochloride. *A. elegans* DSM 11693^T 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.

Primers and PCR. A pair of primers corresponding to *Escherichia coli* 16S rRNA gene positions 8 to 27 (5'-AGAGTTTGATCATGGCTCAG-3') (17) and 1405 to 1391 (5'-ACGGCGGTGTGTAC-3') (8) was obtained from Amersham Pharmacia Biotech (Tokyo, Japan). 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 *Kpn*I in low-salt buffer, then with *Hind*III in medium-salt buffer, and finally with *Pst*I 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 into two *Eco*RI fragments. Their single-stranded DNAs were obtained by subcloning into M13mp18 and sequenced by using a Thermo Sequenase premixed

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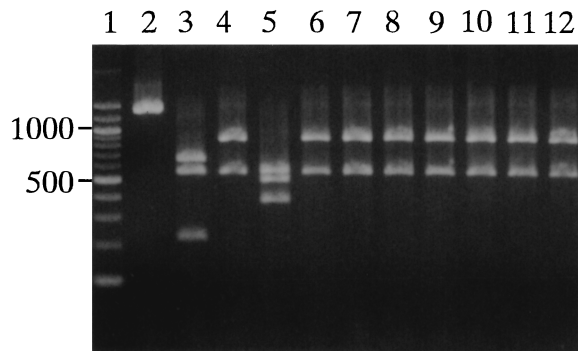


FIG. 1. PCR-RFLP analysis of the 16S rRNA genes. Lanes: 1, size marker of 100-bp ladder; 2, *A. defectiva* ATCC 49176^T; 3, *A. adiacens* ATCC 49175^T; 4, *A. elegans* DSM 11693^T; 5, *G. morbillorum* ATCC 27824^T; 6 to 12, NVS isolates C9-2, HHC5, YTM1, S43-1, NMP3, S943-2, and S1052-1, respectively.

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 DDBJ).

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 [³²P]dCTP (NEN, Boston, Mass.)-labeled DNA in 25 mM phosphate buffer (pH 6.5) containing 30% formamide, 3× SSC (1× 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% sodium dodecyl sulfate (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 0.001% 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'Étoile, France).

DNA sequence data and nucleotide sequence accession numbers. The sequence data for 16S rRNA genes obtained from DDBJ had the following accession numbers: *A. defectiva* ATCC 49176^T, D50541 (7); *A. adiacens* ATCC 49175^T, D50540 (7); *A. elegans* DSM 11693^T, AF016390 (12); *G. morbillorum* ATCC 27824^T, L14327 (18); and *E. coli*, A14565. The sequence of strain HHC5 has been deposited in the DDBJ under accession no. AB022026.

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 not digested 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. adjacens* and four previously identified as *G. morbillorum*. Thus, the 77 isolates of *A. adiacens* consisted of 75 previously identified as *S. adjacens* 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 *Hind*III, *Pst*I, or *Kpn*I sites, *A. adiacens* possessed *Hind*III and *Pst*I sites located at positions 214 and 862, respectively, and *A. elegans* possessed a *Pst*I site located at position 863. Only *G. morbillorum* possessed

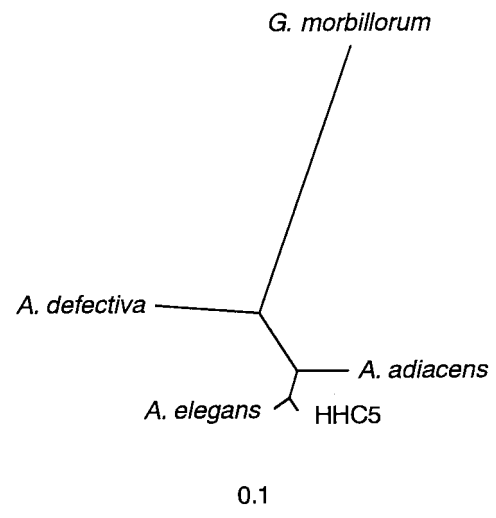


FIG. 2. Phylogenetic relationships based on 16S rRNA gene sequences. The line denotes evolutionary distance.

TABLE 1. DNA-DNA hybridization

Species or strain	Relatedness (%)			
	<i>A. defectiva</i>	<i>A. adiacens</i>	<i>A. elegans</i>	<i>G. morbillorum</i>
<i>A. defectiva</i>	100	6	7	1
<i>A. adiacens</i>	6	100	20	3
<i>A. elegans</i>	6	7	100	1
<i>G. morbillorum</i>	5	4	7	100
HHC5	6	14	70	2

a *KpnI* site at position 492 in addition to a *PstI* site at position 863.

DNA-DNA hybridization. As shown in Table 1, chromosomal DNA of HHC5 hybridized with *A. elegans* DNA at a relatedness of 70%, but it hybridized with *A. defectiva*, *A. adiacens*, and *G. morbillorum* DNAs to a lesser degree.

Biochemical characteristics. We found that arginine dihydrolase (ADH) and urease (URE) were critical to distinguishing *A. elegans* from the other *Abiotrophia* species. As shown in Table 2, ADH was positive in both *A. elegans* DSM 11693^T and the seven related isolates but was negative in all of the others. This suggested that ADH-positive NVS are *A. elegans*. On the other hand, URE was negative in *A. elegans* DSM 11693^T and three of the seven related isolates, but a URE-positive isolate of NVS is probably *A. elegans*, since all of *A. defectiva* and *A. adiacens* and their related clinical isolates were URE negative. The other 30 biochemical characteristics tested by the Rapid ID32 STREP kit did not distinguish *A. elegans* and the seven related isolates from either *A. defectiva* or *A. adiacens*.

Only *A. elegans* DSM 11693^T was unable to grow in the medium supplemented with vitamin B₆ in the absence of L-cysteine, but it did grow in the medium supplemented with L-cysteine in the absence of vitamin B₆, as previously described by Roggenkamp et al. (12). In contrast, all of the other isolates were able to grow in the medium supplemented with either vitamin B₆ or L-cysteine (Table 2). Thus, vitamin B₆ and L-cysteine could be substituted for each other as essential growth factors for all NVS tested with the exception of *A. elegans* DSM 11693^T. We were unable to determine if the requirement for L-cysteine as a growth factor was characteristic of only *A. elegans* DSM 11693^T or of *A. elegans* strains in general.

G. morbillorum did not produce any lytic enzymes and was able to grow in THB containing neither vitamin B₆ nor L-cysteine.

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^T. 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 re-identified 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^T was unable to grow in the vitamin B₆-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

TABLE 2. Critical biochemical characteristics specific to *A. elegans* and seven related isolates

Bacterial strain or isolate (no. of isolates)	Characteristic (no. of isolates)				
	Lytic enzyme production	Vitamin B ₆ ^a	L-Cysteine ^b	Activity	
				ADH	URE
<i>A. defectiva</i> ATCC 49176 ^T	+	+	+	-	-
Clinical isolates identified as <i>A. defectiva</i> (7)	+(7)	+(7)	+(7)	-(7)	-(7)
<i>A. adiacens</i> ATCC 49175 ^T	+	+	+	-	-
Clinical isolates identified as <i>A. adiacens</i> (77)	+(77)	+(77)	+(77)	-(77)	-(77)
<i>A. elegans</i> DSM 11693 ^T	+	-	+	+	-
Clinical isolates identified as <i>A. elegans</i> (7)	+(7)	+(7)	+(7)	+(7)	+(4)
<i>G. morbillorum</i> ATCC 27824 ^T	-	- ^c	- ^c	-	-

^a Ability to grow in vitamin B₆-supplemented medium without L-cysteine.

^b Ability to grow in L-cysteine-supplemented medium without vitamin B₆.

^c *G. morbillorum* required neither vitamin B₆ nor L-cysteine for growth.

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^T 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.

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