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Genetic Heterogeneities and Phenotypic Characteristics of Strains of the Genus *Abiotrophia* and Proposal of *Abiotrophia para-adiacens* sp. nov.

TAISEI KANAMOTO,* SETSUKO SATO, AND MASAKAZU INOUE

Department of Preventive Dentistry, Kagoshima University Dental School, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan

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The genus *Abiotrophia* represents a heterogeneous group of fastidious cocci that show a dependence on pyridoxal hydrochloride analogs for growth. The genetic heterogeneity in the genus *Abiotrophia* was examined by DNA-DNA hybridization, PCR assay of genomic DNA sequences, and restriction fragment length polymorphism and sequence homology analyses of the PCR-amplified 16S rRNA gene. Nine type or reference strains of *Abiotrophia defectiva*, *Abiotrophia adiacens*, and *Abiotrophia elegans* and 36 oral *Abiotrophia* isolates including the ones presumptively identified as *Gemella morbillorum* by the rapid ID32 STREP system were divided into four groups: *A. defectiva* (genotype 1), *A. adiacens* (genotype 2), *A. elegans* (genotype 4), and a fourth species (genotype 3) which we propose be named *Abiotrophia para-adiacens* sp. nov. A PCR assay specific for detection and identification of the novel *Abiotrophia* species was developed. *A. para-adiacens* generally produced β-glucosidase but did not produce α- or β-galactosidase or arginine dihydrolase, did not ferment, trehalose, pullulan, or tagatose, and was serotype IV, V, or VI. Thus, it was distinguished phenotypically from *A. adiacens*, *A. elegans*, and *A. defectiva* as well as, apparently, from the recently described species *Abiotrophia balaenopterae* sp. nov., which produces arginine dihydrolase and which ferments pullulan but not sucrose (P. A. Lawson et al., Int. J. Syst. Bacteriol. 49:503–506, 1999). Strain ATCC 27527, currently listed as *G. morbillorum*, was a member of the species *A. para-adiacens*.

Streptococci that showed satellite growth around colonies of other microorganisms were first described by Frenkel and Hirsch in 1961 (6). They are the normal flora of the human mouth, pharynx, and intestinal and urogenital tracts and are often isolated from patients with so-called culture-negative bacterial endocarditis, bacteremia, and foci of various infectious diseases (2, 13, 16).

These organisms had formerly been known as nutritionally variant streptococci (NVS) and were supposed to be auxotrophic variants of viridans group streptococci (13). NVS require pyridoxal hydrochloride (vitamin B_6) analogs for growth and produce chromophore, pyrrolidonyl arylamidase, and bacteriolytic enzyme in common with some viridans group streptococci (2, 4, 7, 12, 16). However, NVS show considerable variations in their glycosidase and peptidase production profiles (1, 7) and the electrophoresis patterns of their penicillin-binding proteins (4). The species of clinical NVS isolates are often indicated as *Gemella morbillorum* (*Gemella*-like NVS) by the rapid identification systems (5, 7, 21). Four broad biotypes have been demonstrated so far in this unique group of cocci (4, 7).

The earlier DNA-DNA hybridization study divided fastidious NVS into two species, *Streptococcus defectivus* and *Streptococcus adjacens* (2). They were then transferred to the species *Abiotrophia defectiva* and *Abiotrophia adiacens*, respectively, on the basis of 16S rRNA gene sequence homology (8). A third, more fastidious species, *Abiotrophia elegans*, has recently been added to the genus *Abiotrophia* (14, 15). We have found that *A. defectiva*, *A. adiacens*, and *A. elegans* comprise 8, 84, and 8% of the NVS isolates from the human mouth, respectively (17). However, wider genetic heterogeneity among *Abiotrophia* spp. (18) and intraspecies variations in *A. adiacens* (3) have also been suggested.

The molecular genetic approaches such as restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rRNA genes and PCR assay of genomic DNA sequences have become applied to the specific identification and differentiation of *Abiotrophia* spp. (3, 11, 15). In the present study, we examined the genetic variations among 45 *Abiotrophia* strains including the type strains of *A. defectiva*, *A. adiacens*, and *A. elegans* and the oral isolates in our laboratory (7) and propose the presence of a fourth *Abiotrophia* species, *Abiotrophia para-adiacens* sp. nov. During the preparation of this article, a novel species, *Abiotrophia balaenopterae* sp. nov., isolated from a minke whale has been described (10); however, *A. balaenopterae* sp. nov. appears to be distinct from *A. para-adiacens* sp. nov. on the basis of some phenotypic properties that have been reported.

MATERIALS AND METHODS

Bacterial strains. Forty-five *Abiotrophia* strains were used (see Table 1). They included nine type or reference strains of *A. defectiva* (strains ATCC 49176^T, PE7, and NVS-47), *A. adiacens* (strains ATCC 49175^T, C50, L61, G40, and ATCC 27527), and *A. elegans* (strain DSM 11693^T), with all except one known to be isolates from patients with endocarditis or bacteremia (2, 4, 14, 20). Strain ATCC 27527 is a sputum isolate and is listed as *Gemella morbillorum* in the current American Type Culture Collection (ATCC) catalog but was confirmed to be an *A. adiacens* strain by the Rapid ID32 STREP system (Bio Mérieux SA, Marcy-l'Etoile, France) and other bacteriological and biochemical tests described previously (7) (see Results). The other 36 strains were from our laboratory and were isolates from the human mouth (7). The species of most of these strains have been determined and their phenotypes have been characterized, and some were phenotypically characterized and their species were provisionally determined to be *A. defectiva*, *A. adiacens*, or *G. morbillorum* (*Gemella*-like NVS) with the rapid identification system as described previously (7). The strains were biotyped with the emended typing system (17) and were serotyped (K. Kitada, T. Kanamoto, Y. Okada, and M. Inoue, submitted for publication). Strains of the

* Corresponding author. Present address: Department of Microbiology and Immunology, Kagoshima University Dental School, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan. Phone: 81-99-275-6152. Fax: 81-99-275-6158. E-mail: kana@dentb.hal.kagoshima-u.ac.jp.

other six genera and 23 species listed in Table 3 were used for comparisons; of these six species of the genera *Streptococcus*, *Enterococcus*, *Dolosigranurum*, and *Aerococcus* were bacteriolytic and 17 species of the genera *Gemella*, *Streptococcus*, *Aerococcus*, and *Staphylococcus* were nonbacteriolytic. Their *Micrococcus luteus* cell lysis activities were examined as described previously (7).

All the *Abiotrophia* ATCC strains were obtained from ATCC (Manassas, Va.), the DSM strain was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), and the NCFB strains were obtained from the National Collections of Industrial and Marine Bacteria (Aberdeen, United Kingdom). Strains PE7, C50, L61, and G40 were kindly provided by A. Bouvet (Service de Microbiologie, Hôpital Université, Hôtel-Dieu, Paris, France), and strain NVS-47 was provided by I. van de Rijn (Wake Forest University Medical Center, Winston-Salem, N.C.). The *Enterococcus* strains were gifts from T. Takada (Tohoku University, School of Dentistry, Sendai, Miyagi, Japan), and *Streptococcus bovis* strain was from H. Mukasa (Department of Chemistry, National Defence Medical College, Tokorozawa, Saitama, Japan). The other non-*Abiotrophia* strains were from the collections in our laboratory.

Preparation of genomic DNA. *Abiotrophia* strains were normally grown anaerobically at 37°C for 18 h in Todd-Hewitt (TH) broth (Difco, Detroit, Mich.) supplemented with 0.001% pyridoxal hydrochloride. *A. elegans* DSM 11693^T was cultured in TH broth containing 5% horse serum (BioWhittaker, Walkersville, Md.) in place of pyridoxal. The other strains were grown in TH broth. The DNA was extracted from cells and was purified as described previously (19). Briefly, bacterial cells were collected by centrifugation (5,000 × *g*, 15 min), washed three times in saline, and lysed at 37°C for 1 h with egg white lysozyme (30 U/ml; Sigma Chemical Co., St. Louis, Mo.) and mutanolysin (60 U/ml; Sigma) in 0.1× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate). The sample was then incubated at 60°C for 30 min with 0.8% sodium dodecyl sulfate (SDS; Wako Pure Chemical Industries, Tokyo, Japan) in 1× SSC. The extracted DNA was deproteinized with phenol and with a chloroform-isoamyl alcohol (24:1 [vol/vol]) mixture, digested with RNase (0.1 mg/ml), and precipitated with 70% ethanol.

DNA-DNA hybridization. A DNA hybridization probe was labeled with [α-^{32}P] dCTP (111 TBq/mmol; New England Nuclear Research Co., Boston, Mass.) with an oligolabeling kit (Ready-To-Go DNA Labeling Beads; Amersham Pharmacia Biotech, Uppsala, Sweden).

Heat-denatured DNAs (10 μg each) of the test strains were blotted onto a membrane (Hybond-N$^+$; Amersham Pharmacia Biotech) with Immunodot (ATTO Co., Tokyo, Japan) and were fixed by UV cross-linking. The membrane was preincubated in a polyethylene bag at 42°C for 1 h in hybridization solution (30% formamide, 0.45 M NaCl, 45 mM trisodium citrate, 25 mM phosphate buffer [pH 6.5], 5× Denhardt's solution [Wako], 0.2 mg of heat-denatured salmon sperm DNA per ml) and was then incubated with heat-denatured probe DNA for 24 h in the hybridization solution. The membrane was washed in 2× SSC–0.1% SDS at 52°C for 15 min and in 0.2× SSC–0.1% SDS at room temperature for 10 min.

The radioactivity of each blot on the membrane was measured with the BAS1000 bioimaging analyzer system (Fuji Photo Film Co., Tokyo, Japan) according to the unit of radiation dose, photo-stimulated luminescence (PSL), which is proportional to counts per minute. The percent DNA homology for a test strain was calculated by the following equation: 100 × [(test DNA blot PSL − background PSL)/(probe DNA blot PSL − background PSL)].

PCR of genomic DNA sequences. Nucleotide sequences of the 3.3- and 5.7-kb *Kpn*I fragments of strain TKT1 (homology group 3) DNA were determined, and primers specific for the identification of strains of *A. adiacens* DNA homology group 3 (PR1072, 5′-GCGTGAATGCCATCTATCAG-3′ and 5′-ATCCACCA GTCTAAGAACTG-3′) and sequences common to strains of the genus *Abiotrophia* (PR257, 5′-TTATGGTCAGGTGGTAGGAG-3′ and 5′-ACTTGTTGGT CTCGCAGTCA-3′) were synthesized (Amersham Pharmacia Biothch, Tokyo, Japan). Premix Taq (Takara Shuzo Co., Shiga, Japan) containing *Taq* DNA polymerase and PCR buffer (25 μl), template DNA (0.5 μl), and 0.2 μM primers were added to 50 μl of the PCR mixture. PCR was performed for 30 cycles with a profile of 94°C for 1 min and 64°C for 1 min and for an additional extension at 64°C for 10 min with a thermal cycler (Zymoreactor II; ATTO). The PCR products were stained with 2.5% ethidium bromide in an agarose gel. The correct sizes of the amplicons are 1,072 bp for the *A. adiacens* homology group 3-specific PCR and 257 bp for the genus *Abiotrophia*-common PCR.

RFLP analysis of PCR-amplified 16S rRNA gene. Primers (5′-AGAGTTTG ATCATGGCTCAG-3′ and 5′-ACGGGCGGTGTGTAC-3′) which correspond to the *Escherichia coli* 16S rRNA gene (positions 8 to 17 and 1405 to 1391, respectively; Amersham Pharmacia Biotech) were used to amplify the 16S rRNA genes of the test strains (9). PCR was carried out as described above, and the amplicon obtained was digested at 37°C for 40 min with 5 U each of the restriction enzymes, *Eco*RI, *Xba*I, and *Hin*dIII (Nippon Gene, Osaka, Japan). Profiles of the digests were analyzed in a 2.5% agarose gel after staining with ethidium bromide.

16S rRNA gene sequence. The PCR product was cloned into a pCR2.1 vector (TA cloning kit; Invitrogen Corporation, Carlsbad, Calif.) and was cut into two *Eco*RI fragments. Their single-stranded DNAs were obtained by subcloning into M13mp18 and were sequenced by using a Thermo Sequenase premixed cycle sequence kit (Amersham) with an automatic sequencer (SQ5500; Hitachi Ltd., Tokyo, Japan). The sequence of strain TKT1 was compared with those of the other related strains obtained from the data deposited in the DNA Data Bank of Japan (DDBJ; Mishima, Shizuoka, Japan) and was analyzed to construct a phylogenetic tree by using DDBJ Super Computer (VPP500; Fujitsu, Tokyo, Japan) and the program ClustalW (supplied by DDBJ).

Nucleotide sequence accession numbers. DNA sequence data for the 16S rRNA genes obtained from DDBJ had the indicated accession numbers: *A. defectiva* ATCC 49176^T, D50541; *A. adiacens* ATCC 49175^T, D50540; *A. elegans* DSM 11693^T, AF016390; *G. morbillorum* ATCC 27824^T, L14327; and *E. coli*, A14565. The sequence of strain TKT1 has been deposited in DDBJ under accession no. AB022027.

RESULTS

DNA homology. By using the probe DNAs prepared from two strains each of NVS biotypes 1 to 4 (emended) (7, 17), the 45 strains tested were divided into four homology groups, although DNAs from a few homology group 3 and 4 strains gave low percent homologies (less than 50%) with all eight probe DNA preparations used (Tables 1 and 2). Homology group 1 strains were clearly distinguished from strains of the other groups. Homology group 2 and 3 strains were also separated, although their DNA homology levels were not so distantly related to each other; e.g., the mean ± standard deviation homologies were 75.7% ± 18.2% and 50.1% ± 16.9%, respectively, when the strains were probed with ATCC 49175 DNA. Homology group 4 strains constituted an independent group. In this group, DNA of *A. elegans* DSM 11693^T showed a low degree of homology (23.3%) when its DNA was hybridized with the HHC5 probe DNA, but the HHC5 DNA had a degree of high homology (70.7%) with the DSM 11693 probe DNA. Mean homology values for these two probe DNAs for the homology group 4 strains were very similar (63.6 versus 61.3%; Table 2).

Strain ATCC 27527 (homology group 3), which is listed as *G. morbillorum* in the current ATCC catalog, was revealed to be dependent on pyridoxal for growth and to produce chromophore and bacteriolytic enzyme, and its species was determined to be *A. adiacens*, although at a rather low confidence level, by the Rapid ID32 STREP system. The strain was not genetically related to the pyridoxal-independent, nonlytic, bona fide strain *G. morbillorum* ATCC 27824^T (Table 1). DNAs from the bacteriolytic but pyridoxal-independent strains *Dolosigranurum pigrum* NCFB 2975^T and *Aerococcus urinae* NCFB 2893^T did not react with any one of the eight *Abiotrophia* DNA probes.

PCR of genomic DNA sequences. The genetic variations among the *Abiotrophia* strains described above, particularly the differentiation of homology group 3 strains from the other strains, were confirmed by PCR-based assays.

The 5.7-kb fragment of *Kpn*I-digested genomic DNA of strain TKT1 (homology group 3) hybridized with DNAs of all *Abiotrophia* strains, and the 3.3-kb *Kpn*I fragment hybridized only with DNA of homology group 3 strains (data not shown). The base sequences of these fragments were determined, and two sets of PCR primers specific for differentiation of *Abiotrophia* species, primers PR257 and PR1072, were designed. The product of PCR with primer PR1072 was detected only with the homology group 3 strains, and the product of PCR with primer PR257 was detected with all homology group 1 to 4 strains (Table 3). Thus, in the profile for the product of PCR with primer PR1072, *A. adiacens* homology group 2 and 3 strains were clearly distinguished from each other and were separated from *A. elegans* homology group 4 and *A. defectiva* homology group 1 strains.

Furthermore, the PCR assay with PR1072 was negative for strains of the other six genera and 23 species examined, irrespective of whether they were bacteriolytic or nonbacteriolytic (Table 3). In contrast, the PCR assay with PR257 was positive

TABLE 1. DNA homologies of *Abiotrophia* strains

Strain	Species determined by Rapid ID32 STREP system[a]	Emended NVS biotype[a,b]	% Homology with the probe DNA from:				
			A. defectiva ATCC 49176^T	*A. adiacens* ATCC 49175^T	*A. adiacens* TKT1	*Gemella*-like NVS HHC5	*G. morbillorum* ATCC 27824^T
Abiotrophia spp.							
Homology group 1							
ATCC 49176^T	Ad	1	100.0	6.2	5.0	4.8	0.9
NVS-47	Ad	1	98.0	6.8	7.9	4.9	—[c]
YK-3	Ad	1	90.2	5.1	4.6	5.4	—
TK-1	Ad	1	71.7	4.6	3.4	4.9	—
PE7	Ad	1	63.7	3.5	2.6	3.6	—
C8-3	Ad	1	62.8	18.7	30.1	7.5	—
TK-4	Ad	1	57.6	5.0	2.8	4.5	—
S1057-1	Ad	1	57.6	5.2	4.0	3.7	—
YTS2	Ad	1	55.2	4.1	3.6	3.1	—
Homology group 2							
ATCC 49175^T	Aa	2	6.1	100.0	30.7	11.1	3.2
C50	Aa	2	3.2	116.2	60.5	29.1	—
C6-2	Aa	3	2.4	84.6	55.4	22.8	—
HHC3	Aa	2	5.5	82.7	32.1	10.6	—
L61	Aa	3	8.6	81.8	44.5	14.5	—
YTC1	Aa	2	4.7	81.3	31.2	11.1	—
C1-3	Aa	3	1.8	80.9	41.7	20.1	—
S961-2	Aa	2	7.8	80.2	36.8	14.9	—
C2-1	Aa	3	1.6	79.5	34.5	18.5	—
C1-1	Aa	2	2.8	65.9	61.8	20.9	—
P6-1	Aa	3	4.5	64.4	27.0	9.7	—
G40	Aa	3	4.9	60.3	20.8	8.7	6.2
HHP1	Aa	2	3.1	55.6	19.0	7.3	—
S1058-2	Aa	3	4.8	55.6	22.5	7.7	—
C2-2	Aa	2	3.7	46.1	23.0	6.2	—
Homology group 3							
TKT1	Aa	2	9.5	58.7	100.0	14.9	14.9
S49-2	Aa	2	8.8	74.4	84.8	17.4	—
TKT2	Gm	3	5.9	43.2	83.2	10.6	10.3
NMP2	Aa	3	9.4	75.7	81.0	19.4	—
C4-1	Aa	3	2.6	49.0	77.9	21.0	—
HKT1-4	Aa	3	8.3	62.0	71.5	16.6	—
C5-1	Aa	2	2.7	51.4	70.7	19.1	—
HKT1-1	Gm	3	9.2	60.1	69.0	17.8	9.5
C4-3	Aa	2	6.0	43.7	57.7	10.5	6.9
P7-4	Aa	2	7.0	53.3	53.3	14.7	—
ATCC 27527	Aa	3	4.8	33.4	40.3	8.3	3.3
HKT2-2	Aa	3	4.0	25.9	32.2	7.9	—
YTT3	Aa	2	3.1	20.5	22.2	4.8	—
Homology group 4							
HHC5	Gm	4	5.9	14.4	12.4	100.0	1.7
S43-1	Gm	4	2.7	30.0	24.0	85.2	—
YTM1	Gm	4	5.5	16.1	10.8	83.5	2.4
C9-2	Gm	4	4.9	11.7	8.8	80.7	2.9
NMP3	Aa	4	4.0	11.7	8.1	55.4	—
S943-2	Aa	4	3.7	9.4	6.4	41.6	—
S1052-1	Aa	4	4.2	8.0	5.3	38.8	—
DSM 11693^T	Aa	4	5.8	7.3	7.0	23.3	
G. morbillorum ATCC 27824^T	Gm	—	5.2	4.1	4.1	3.8	100.0
D. pigrum NCFB 2975^T	—	—	4.9	3.4	2.5	3.6	1.1
A. urinae NCFB 2893^T	—	—	2.3	0.5	0.2	0.9	4.0

[a] From reference 7; Ad, *A. defectiva*; Aa, *A. adiacens*; Gm, *G. morbillorum*.
[b] From reference 17.
[c] —, not determined.

TABLE 3. PCR products obtained with primers specific for *Abiotrophia* genotypes

Strain	No. of strains tested	No. of strains positive for products by PCR with the following primers:	
		PR1072	PR257
Abiotrophia spp.			
Homology group 1	9	0	9
Homology group 2	15	0	15
Homology group 3	13	13	13
Homology group 4	8	0	8
Other spp.			
Bacteriolytic			
Streptococcus intermedius ATCC 27335^T		0	1
Enterococcus durans ATCC 19432^T		0	1
Enterococcus faecalis SS499		0	1
Enterococcus hirae ATCC 9790		0	1
D. pigrum NCFB 2975		0	0
A. urinae NCFB 2893		0	0
Nonbacteriolytic			
G. morbillorum ATCC 27824^T		0	0
Gemella haemolysans ATCC 10378^T		0	0
Aerococcus viridans DSM 20340		0	1
Streptococcus mitis ATCC 6249		0	1
Streptococcus oralis ATCC 9811		0	1
Streptococcus sanguinis ATCC 10556^T		0	1
Streptococcus gordonii ATCC 10558^T		0	1
Streptococcus parasanguinis ATCC 15909		0	1
Streptococcus anginosus ATCC 33397^T		0	1
Streptococcus constellatus NCTC 10708		0	1
Streptococcus cricetus ATCC 19642^T		0	1
Streptococcus ratti ATCC 19645^T		0	1
Streptococcus mutans ATCC 33535^T		0	1
Streptococcus sobrinus OMZ176		0	0
Streptococcus salivarius ATCC 13419^T		0	0
Streptococcus bovis ATCC 33317^T		0	0
Staphylococcus aureus ATCC 21027		0	1

for non-*Abiotrophia* strains (four genera and 16 species). These PCR assays were negative for four genera and seven species, including bona fide strain *G. morbillorum* ATCC 27824^T.

16S rRNA gene PCR-RFLP analysis. Genetic heterogeneities were examined further by use of the 16S rRNA gene PCR-RFLP profiles of the DNA homology groups. All strains of *A. adiacens* homology group 2 (strains ATCC 49175^T, L61, C1-3, and C2-2) and group 3 (strains TKT1, TKT2, C4-1, HKT1-1, and ATCC 27527) tested gave four fragments of 0.21, 0.34, 0.40, and 0.46 kb. In contrast, strains of *A. elegans* homology group 4 (strains HHC5, YTM1, and DSM 11693^T) as well as strains of *A. defectiva* homology group 1 (strains ATCC 49176^T, PE7, and C8-3) had three fragments of 0.34, 0.40, and 0.68 kb. Thus, the four homology groups were not satisfactorily discriminated from each other by the PCR-RFLP analysis in this study, but the 16S rRNA gene of *G. morbillorum* ATCC 27824^T gave a unique profile with three fragments of 0.26, 0.48, and 0.68 kb.

16S rRNA gene sequence homology. The TKT1 16S rRNA gene sequence was 1,407 bp in length and included the forward and reverse primers in the 5′ and 3′ directions, respectively. A multiple alignment analysis showed that the sequence homologies between TKT1 and the type strains of *A. adiacens*, *A. elegans*, *A. defectiva*, and *G. morbillorum* were 99, 97, 92, and 85%, respectively. As depicted in an unrooted phylogenetic

TABLE 2. Mean homology with various probe DNAs

Homology group	% Homology (mean ± SD) with the probe DNA from:								
	A. defectiva ATCC 49176^T (1)[a]	*A. defectiva* NVS-47 (1)	*A. adiacens* ATCC 49175^T (2)	*A. adiacens* TKT1 (2)	*A. adiacens* ATCC 27527 (3)	*A. adiacens* NMP3 (3[b])	*Gemella*-like NVS HHC5 (4[b])	*A. elegans* DSM 11693^T (4[b])	*G. morbillorum* ATCC 27824^T
Abiotrophia spp.									
Homology group 1	73.0 ± 18.1[c] (9)[d]	*62.1 ± 19.2[c] (8)	6.6 ± 4.7 (9)	7.1 ± 8.8 (9)	5.0 ± 1.2 (8)	5.8 ± 1.2 (8)	4.7 ± 1.3 (9)	6.6 ± 1.6 (8)	0.9 (1)
Homology group 2	4.4 ± 2.0 (15)	4.8 ± 1.4 (10)	75.7 ± 18.2[c] (15)	36.1 ± 14.1 (15)	32.5 ± 10.6 (10)	14.7 ± 4.7 (10)	14.2 ± 6.7 (15)	18.4 ± 5.9 (10)	4.7 ± 2.1 (2)
Homology group 3	6.3 ± 2.6 (13)	6.8 ± 2.6 (11)	50.1 ± 16.9 (13)	64.9 ± 22.7[c] (13)	73.7 ± 26.9[c] (11)	18.0 ± 6.3 (11)	14.1 ± 5.1 (13)	23.1 ± 7.6 (11)	9.0 ± 4.3 (5)
Homology group 4	4.6 ± 1.1 (8)	3.6 ± 0.8 (7)	13.6 ± 7.3 (8)	10.4 ± 6.0 (8)	8.6 ± 2.6 (7)	70.1 ± 28.7[c] (7)	63.6 ± 27.4[c] (8)	61.3 ± 22.3[c] (7)	1.9 ± 0.6 (4)
G. morbillorum ATCC 27824^T	5.2 (1)	4.2 (1)	4.1 (1)	4.1 (1)	4.5 (1)	5.7 (1)	3.8 (1)	7.2 (1)	100.0 (1)

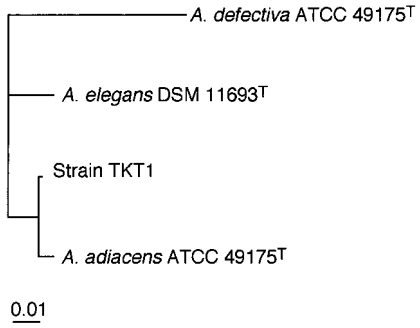

FIG. 1. The 16S rRNA gene sequence-based phylogenetic distance between strain TKT1 (DNA homology group 3) and *A. defectiva* (homology group 1), *A. adiacens* (homology group 2), and *A. elegans* (homology group 4).

tree (Fig. 1), DNA homology group 3 strain TKT1 was most closely related to *A. adiacens* ATCC 49175^T (homology group 2) and was considerably more distantly related to the other *Abiotrophia* species (homology groups 4 and 1), and all the *Abiotrophia* type strains were remote in terms of their phylogenetic distance from *G. morbillorum* ATCC 27824^T.

Phenotypic characteristics of *Abiotrophia* genotypes. As described above, the genus *Abiotrophia* was differentiated into four genetic categories (designated genotypes 1 to 4). The physiological and serological properties of strains of these genotypes are summarized from the data reported previously (7, 17; Kitada et al.; submitted) (Table 4).

The genotype 1 strains were restricted to *A. defectiva* biotype 1 (7): they were distinctly differentiated from the other strains by production of α- and β-galactosidases but a lack of production of either β-glucosidase or β-glucuronidase, fermentation of trehalose and pullulan, and being of serotype I. In contrast, the genotype 4 strains belonged to *A. adiacens* or *Gemella*-like biotype 4 (emended) (17). They all produced arginine dihydrolase specifically and usually produced β-glucosidase and/or *N*-acetyl-β-glucosaminidase. All except one of the genotype 4 strains were serotype VII or VIII. Genotypes 2 and 3 were also mixtures of *A. adiacens* biotype 2 and 3 (emended) strains. The genotype 2 strains generally produced β-glucosidase and *N*-acetyl-β-glucosaminidase and fermented tagatose. Generally, the genotype 3 strains also produced β-glucosidase but did not ferment tagatose. The genotype 2 strains were serotype II or III, whereas the genotype 3 strains were serotype IV, V, or VI or untypeable. Thus, these two genotypes were also phenotypically distinguishable.

DISCUSSION

The *Abiotrophia* spp. are known to be pyridoxal dependent and to produce chromophore, pyrrolidonyl arylamidase, and

TABLE 4. Phenotypic characteristics of strains of *Abiotrophia* genotypes

Characteristic	No. of strains			
	Genotype 1, *A. defectiva* (n = 9)[a]	Genotype 2, *A. adiacens* (n = 15)	Genotype 3, *A. para-adiacens* (n = 13)	Genotype 4, *A. elegans* (n = 8)
Biotype[b]				
Biotype 1	9	0	0	0
Biotype 2	0	8	6	0
Biotype 3	0	7	7	0
Biotype 4	0	0	0	8
Pyridoxal dependency for growth	9	15	13	8
Chromophore production	9	15	13	8
Bacteriolytic enzyme production	9	15	13	8
Production of:				
α-Galactosidase	9	0	0	0
β-Galactosidase	9	0	0	0
β-Glucosidase	0	13	10	3
N-Acetyl-β-glucosaminidase	2	12	6	3
β-Glucuronidase	0	8	6	0
Fermentation of:				
Trehalose	9	0	0	0
Pullulan	9	0	0	0
Tagatose	4	11	2	0
Sucrose	9	15	13	8
Arginine dihydrolase	0	0	0	8
Serotype[c]				
I	9	0	0	0
II, III	0	14	2	0
IV, V, VI	0	1	8	0
VII, VIII	0	0	0	7
untypeable	0	0	3	1

[a] n is number of strains tested.

[b] By the emended definition (17), biotype 1 is α- and β-galactosidase production but β-glucuronidase nonproduction; biotype 2 is α- and β-galactosidase nonproduction but β-glucuronidase production; biotype 3 is β-glucuronidase nonproduction but generally *N*-acetylglucosamine production; biotype 4 is α- and β-galactosidase and β-glucuronidase nonproduction but arginine dihydrolase production, irrespective of the identification as *G. morbillorum* by the Rapid ID32 STREP system (7).

[c] Determined with autoclaved extracts of whole cells with the eight serotyping antisera by immunodiffusion in an agar gel (Kitada et al., submitted).

bacteriolytic enzyme (2, 4, 7, 12, 16), but it has been known that the genus *Abiotrophia* represents a heterogeneous group of bacteria (1, 4, 15, 18) and three species and four biotypes of this unique genus have long been known (4, 7, 17). Very recently, during the preparation of this article, a new species has been added to the genus *Abiotrophia* (10).

The present studies demonstrated that the genus *Abiotrophia* is divided into four genetic groups. DNA-DNA hybridization analysis (Table 1) and analysis of the PCR product profiles of the genomic DNA and 16S rRNA gene sequences (Fig. 1; Table 3) differentiated the genotype 3 strains from the other strains and separated most strains of *A. adiacens* identified phenotypically (7) into genotypes 2 and 3, although the two genotypes appear to be rather closely related to each other. These results collectively indicate that along with *A. defectiva* (genotype 1), *A. adiacens* (genotype 2), and *A. elegans* (genotype 4), an additional *Abiotrophia* species (genotype 3) exists, and that it is most closely related to but distinct from *A. adiacens* (genotype 2). We propose here that *Abiotrophia* genotype 3 be called *Abiotrophia para-adiacens* sp. nov.

A. balaenopterae sp. nov., which was very recently described, was recovered from the lung of a minke whale and has also been shown to be closely related to but distinct from *A. adiacens* and *A. elegans* (10). The novel *Abiotrophia* species ferments pullulan but not sucrose and produces arginine dihydrolase and thus appears to be distinguished from *A. para-adiacens* which was segregated from *A. adiacens* (Table 4). The genetic relation between these two new species should be determined in further studies.

The *A. para-adiacens* (genotype 3) strains constituted a large cluster almost equal in size to the *A. adiacens* (genotype 2) cluster (Table 1). Both of these species, however, were mixture of biotype 2 and 3 (emended) strains (Tables 1 and 4) and were rather difficult to differentiate by the tests in the rapid identification system. However, the confidence level in the identification of the strain as *A. adiacens* by the Rapid ID32 STREP system was "excellent" to "good" for most of the genotype 2 strains (80.0%), whereas it was "low" to "id not valid" for most genotype 3 strains (76.9%) (7). In addition, so far tagatose fermentation appears to be a putative key property for the differentiation of these two species: generally, the *A. adiacens* strains produced β-glucosidase and/or fermented tagatose, while the *A. para-adiacens* strains produced β-glucosidase and/or did not ferment tagatose. Moreover, most strains of the former species were serotype II or III, but those of the latter species were serotype IV, V, or VI (Table 4). In fact, all except a few serotype II or III strains maintained in our laboratory (39 strains) ferment tagatose (89.7%), whereas only a few tagatose-positive but serotype IV, V, or VI strains (9.4%) are found in *A. para-adiacens* (Kitada et al., submitted).

In contrast, the remaining two *Abiotrophia* species were phenotypically distinct. *A. elegans*, which consisted of emended biotype 4 strains (17) of *A. adiacens* or *Gemella*-like NVS (7), was clearly separated from the other *Abiotrophia* species in that it produced arginine dihydrolase. *A. defectiva* corresponded strictly to NVS biotype 1 (7) and was also distinct from the others by the production of α- and β-galactosidases and the fermentation of trehalose (4, 7) (Table 4).

Not many *A. elegans* strains have so far been isolated (14, 15). They occupy ca. 10% or less of the clinical *Abiotrophia* isolates in our laboratory (17). *A. elegans* is reported to be more fastidious than the other *Abiotrophia* spp. and to show unique nutritional requirements: it does not use pyridoxal hydrochloride but uses L-cysteine in TH broth as a growth factor (14). In fact, unlike the other genotype 4 strains, the DSM 11693^T substrain that we maintain cannot grow in TH broth and other complex media supplemented with vitamin B_6 instead of horse serum (preliminary results). However, the substrain and the genotype 4 oral isolates resembled each other genetically, biochemically, and serologically (Tables 1, 3, and 4), thus forming a homologous cluster, *A. elegans*. Strains of the other *Abiotrophia* spp. did not hydrolyze arginine but *A. elegans* strains did hydrolyze arginine (17) (Table 4). The existence of an atypical *A. adiacens* strain which produces arginine dihydrolase has been reported (18), and *A. elegans* has been known to be genetically most closely related to *A. adiacens* (14, 17).

Strain ATCC 27527, known as *G. morbillorum*, was identified as *A. adiacens* by the rapid identification system (Table 1), showed phenotypic characteristics typical of those of *Abiotrophia* spp. (Table 4), and, together with some *Gemella*-like NVS and *A. adiacens* strains (7), belonged to genotype 3 and was not related to *G. morbillorum* ATCC 27824^T (Tables 1 and 3). Thus, strain ATCC 27527 is certainly a member of *A. para-adiacens* genotype 3. The genera *Abiotrophia* and *Gemella* are shown to be located far apart in genetic distance, as estimated by comparison of the 16S rRNA gene sequences (8, 17), but *Abiotrophia* strains are occasionally identified as *G. morbillorum* by the rapid identification tests (7) (Table 1) and are misidentified, as in the case of strain ATCC 27527 in the present study.

The PCR-based molecular approaches such as RFLP analysis of universal 16S rRNA PCR products have been attempted for the identification and differentiation of *Abiotrophia* spp. (8, 11, 15). Primer PR1072 was specific for the separation of *A. para-adiacens* from *A. adiacens* as well as *A. defectiva* and *A. elegans*. The PCR assay with primer PR1072 was negative for the nonbacteriolytic and bacteriolytic strains of the other six genera and 23 species tested, including 13 species of oral viridans group streptococci and 10 species including a bona fide *G. morbillorum* strain (Table 3). Thus, the PCR assay with PR1072 would facilitate the specific and sensitive identification of *A. para-adiacens.*

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