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Development of Amplified 16S Ribosomal DNA Restriction Analysis for Identification of *Actinomyces* Species and Comparison with Pyrolysis-Mass Spectrometry and Conventional Biochemical Tests

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Identification of *Actinomyces* spp. by conventional phenotypic methods is notoriously difficult and unreliable. Recently, the application of chemotaxonomic and molecular methods has clarified the taxonomy of the group and has led to the recognition of several new species. A practical and discriminatory identification method is now needed for routine identification of clinical isolates. Amplified 16S ribosomal DNA restriction analysis (ARDRA) was applied to reference strains ($n = 27$) and clinical isolates ($n = 36$) of *Actinomyces* spp. and other gram-positive rods. Clinical strains were identified initially to the species level by conventional biochemical tests. However, given the low degree of confidence in conventional methods, the findings obtained by ARDRA were also compared with those obtained by pyrolysis-mass spectrometry. The ARDRA profiles generated by the combination of *Hae*III and *Hpa*II endonuclease digestion differentiated all reference strains to the species or subspecies level. The profiles correlated well with the findings obtained by pyrolysis-mass spectrometry and by conventional tests and enabled the identification of 31 of 36 clinical isolates to the species level. ARDRA was shown to be a simple, rapid, cost-effective, and highly discriminatory method for routine identification of *Actinomyces* spp. of clinical origin.

The genus *Actinomyces* comprises a heterogeneous group of anaerobic and facultatively anaerobic, asporogenous, nonmotile, non-acid-fast, gram-positive rods with a G+C content of 55 to 71 mol% (2). Many *Actinomyces* spp. are known to be indigenous to mucous membranes, particularly those in the oral cavity, in humans and other animals. Members of the genus are known to cause actinomycosis and may be found in polymicrobial infections arising from tissue invasion by oral anaerobes (16). Some species are significant in periodontal disease (16).

Detection of the presence of *Actinomyces* spp. in clinical specimens may affect the prognosis and patient management, but identification by conventional phenotypic methods is notoriously difficult and unreliable (6). Problems arise from the slow and granular growth of some isolates (particularly *Actinomyces israelii*), the poor reproducibilities of biochemical tests, and the lack of discriminatory power of biochemical tests. The lack of discriminatory power may indicate heterogeneity within species. Indeed, for some species, subdivisions based on biochemical or serological differences have been described (17, 18), and combination in a single genospecies of some serotypes of *Actinomyces naeslundii* and *Actinomyces viscosus* has been proposed (8).

Recently, the application of chemotaxonomic and molecular methods has clarified the taxonomy of the group and has led to the recognition of several new species. These include *Actinomyces europaeus* (4), *Actinomyces graevenitzii* (12), *Actinomyces neuii* (5), *Actinomyces radingae* (22), and *Actinomyces turicensis* (22). Other new species have been assigned to other, similar genera: *Arcanobacterium bernardiae* (13), *Arcanobacterium phocae* (13), *Arcanobacterium pyogenes* (13), *Actinobaculum schaalii* (9), and *Actinobaculum suis* (9). Currently, little is known of the natural habitats, clinical prevalence, and pathogenic potential of these species. A discriminatory, reproducible, and practical method for characterization of clinical isolates may help elucidate their occurrence and significance.

Pyrolysis mass-spectrometry (PMS) has been shown to be a useful tool in differentiating groups of organisms to the species or subspecies level (10) and represents a whole-cell analysis approach independent of conventional biochemical tests. However, PMS is a fingerprinting method and is best suited to examination of large batches of organisms. Barsotti et al. (1) demonstrated the potential of rRNA gene (rDNA) restriction patterns as taxonomic tools for *Actinomyces*. This approach may be enhanced by initial amplification of rDNA by PCR. Amplified rDNA restriction analysis (ARDRA) has been used to identify various fungi (21) and bacteria (19, 20).

In this study, ARDRA was applied to reference strains and clinical isolates of *Actinomyces* spp. and some other gram-positive rods. Clinical isolates were identified initially to the species level by conventional biochemical tests and comparison of the results with those for reference strains. However, given the low degree of confidence in this method, the findings were also compared with those obtained by PMS.

MATERIALS AND METHODS

Bacterial strains. A total of 63 strains were examined (Table 1). Reference strains ($n = 27$) represented 17 species of *Actinomyces* and 5 species of other, similar genera. Clinical strains comprised 35 human isolates and 1 animal isolate from the collection held by the Anaerobe Reference Unit, Public Health Laboratory Service. Strains were selected to represent the range of species and sites

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TABLE 1. Sources of bacterial strains[a]

Clinical isolates			Reference strains[b]		
Strain	Organism	Source	Strain	Organism	Reference
VH20	*A. denticolens*	Abdominal abscess	VH45	*A. israelii*	ATCC 12102
VH29	*A. georgiae*	Cerebral abscess	VH34	*A. israelii*	NCTC 10236
VH24	*A. georgiae*	IUCD	VH28	*A. gerencseriae*	ATCC 23860
VH48	*A. gerencseriae*	Facial abscess	VH14	*A. naeslundii* serotype I	NCTC 10301
VH16	*A. gerencseriae*	Eyelid puncta	VH55	*A. naeslundii* serotype II	ATCC 44339
VH4	*A. gerencseriae*	Jaw abscess	VH56	*A. naeslundii* serotype III	ATCC 44340
VH32	*A. gerencseriae*	Vertebral abscess	VH36	*A. viscosus* serotype I	NCTC 10951
VH15	*A. israelii*	Pleural pus	VH54	*A. viscosus* serotype II	ATCC 27044
VH5	*A. israelii*	IUCD	VH6	*A. odontolyticus*	NCTC 09935
VH1	*A. israelii*	Mandibular sinus	VH10	*A. meyeri*	ATCC 35568
VH38	Most like *A. israelii*	Perinephric abscess	VH11	*A. georgiae*	ATCC 49285
VH21	*A. israelii*	IUCD	VH47	*A. denticolens*	NCTC 11490
VH44	*A. meyeri*	IUCD	VH43	*A. slackii*	NCTC 11923
VH35	*A. meyeri*	IUCD	VH25	*A. bovis*	NCTC 11535
VH22	*A. meyeri*	Groin abscess	VH7	*Actinomyces hordeovulneris*	ATCC 35275
VH41	*A. meyeri*	Peritoneal pus	VH30	*A. howellii*	NCTC 11636
VH12	*A. meyeri*	Pleural effusion	VH51	*A. graevenitzii*	CCUG 27294
VH8	*A. naeslundii*	Gingival swab	VH52	*A. europaeus*	CCUG 32789A
VH26	Most like *A. naeslundii*	Blood culture	VH57	*A. neuii* subsp. *neuii*	DSM 8576
VH42	Most like *A. naeslundii*	Parotid duct	VH58	*A. neuii* subsp. *anitratus*	DSM 8577
VH2	*A. naeslundii*	IUCD	VH60	*A. turicensis*	DSM 9168
VH39	*A. naeslundii*	IUCD	VH61	*A. radingae*	DSM 9169
VH27	Most like *A. odontolyticus*	Jaw pus	VH53	*A. schaalii*	CCUG 27420
VH50	Most like *A. odontolyticus*	Liver abscess	VH63	*A. suis*	DSM 20639
VH13	*A. odontolyticus*	Oral bone plate	VH19	*A. pyogenes*	NCTC 05224
VH40	*A. odontolyticus*	Blood culture	VH59	*A. bernardiae*	DSM 9152
VH33	Most like *A. odontolyticus*	IUCD	VH62	*A. phocae*	DSM 10002
VH37	Most like *A. pyogenes*	Cow jaw			
VH18	*A. viscosus*	Submandibular abscess			
VH3	Most like *A. viscosus*	Groin			
VH49	Most like *A. viscosus*	IUCD			
VH46	Most like *A. viscosus*	Fractured mandible			
VH23	Most like *A. viscosus*	IUCD			
VH17	Most like *A. viscosus*	Lacrimal fluid			
VH9	*Actinomyces* species	Dental abscess			
VH31	*Actinomyces* species	Osteomyelitis			

[a] Abbreviations: IUCD, intrauterine contraceptive device; ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, United Kingdom; CCUG, Culture Collection, University of Goteborg, Goteborg, Sweden; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

[b] Reference strains other than *A. israelii* NCTC 10236 represent type strains for species or subspecies.

of isolation of strains referred for identification from laboratories throughout England and Wales. Strains were stored at −80°C on Microbank beads (Pro-lab Diagnostics, Wirral, United Kingdom) and were recovered on Fastidious Anaerobe Agar (FAA; LabM, Bury, United Kingdom) incubated anaerobically at 37°C for 48 h.

Conventional tests. Strains were determined to be members of the genus *Actinomyces* on the basis of volatile and nonvolatile fatty acid end products of glucose metabolism, as detected by gas-liquid chromatography as described elsewhere (7).

Cell and colonial morphologies, pigment production, fluorescence under longwave UV illumination, ability to grow in air and in air plus 5% CO_2, and production of catalase and indole were recorded. Hydrolysis of esculin and starch and production of acid from amygdalin, arabinose, cellobiose, glucose, mannitol, raffinose, ribose, salicin, sucrose, trehalose, and xylose were tested by the method of Phillips (14). Production of nitrate reductase, urease, pyrazinamidase, β-galactosidase, α-glucosidase, and β-*N*-acetyl-glucosaminidase was detected after incubation for 18 to 24 h with Rosco diagnostic tablets (BioConnections, Leeds, United Kingdom). Identifications were made initially by reference to the scheme of Brazier and Hall (3) and subsequently by reference to the publications of Funke et al. (5), Pascual Ramos et al. (12), Funke et al. (4) and Lawson et al. (9).

PMS. Pyrolysis was performed with colonies from 48-h anaerobic FAA cultures, and the mass spectra were analyzed as described elsewhere (11).

ARDRA. All strains were tested in triplicate. Strains were cultured anaerobically on FAA for 48 h.

For DNA extraction, a 1-μl loopful of bacteria was suspended in 100 μl of Chelex resin (5%; Bio-Rad Laboratories, Richmond, Calif.) in sterile distilled water, boiled for 8 min, and centrifuged at 17,000 × *g* for 10 min. The supernatant was decanted.

For PCR, DNA extract (5 μl) was added to 45 μl of a reaction mixture containing 1 U of *Taq* polymerase (Pharmacia Biotech, St. Albans, United Kingdom), each deoxynucleoside triphosphate at a concentration of 200 mM, 20 pmol of each primer (pA and pH′), 2.25 mM magnesium chloride, 10 mM Tris HCl (pH 9), 50 mM KCl, and 0.1% Triton X-100. The primer sequences were AGAGTTTGATCCTGGCTCAG (pA) and AAGGAGGTGATCCAGCCGCA (pH′). Amplification was accomplished by 31 cycles of denaturation at 92°C for 2 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, with a final extension period of 5 min. The specific PCR product (approximately 1,600 bp) was detected by electrophoresis of a 5-μl sample for 40 min at 5 V/cm in 1% agarose in TAE (Tris-acetate-EDTA) buffer with ethidium bromide (0.5 μg/ml) and was visualized under UV illumination.

Restriction endonuclease digestion was performed with *Hae*III and *Hpa*II endonucleases in separate reaction mixtures containing 1 μl of endonuclease (10 U/μl), 1.5 μl of matched incubation buffer, and 12.5 μl of PCR product; the mixture was incubated for 1.5 h at 37°C. For electrophoresis, 3 μl of 6× loading buffer (Advanced Biotechnologies, Epsom, United Kingdom) was added to each sample, and 15 μl was electrophoresed for 1.75 h at 5 V/cm in Metaphor agarose (3.5%; FMC, Rockland, Maine) in TAE buffer with ethidium bromide (0.5 μg/ml). Molecular size markers (2 kb; Sigma, Poole, United Kingdom) were run in duplicate or triplicate alongside the samples. The gels were destained in deionized water for 10 min and photographed under UV illumination, and the images were stored on a floppy disk.

Gel data were analyzed with GelCompar software (Applied Maths, Kortrijk, Belgium). Distinct banding patterns were assigned three-digit codes, arbitrarily numbered in the order encountered, and were stored as types in HAE and HPA libraries. The combined results for each strain were recorded as, for example, 001/003, which represent the *Hae*III and *Hpa*II profiles, respectively.

TABLE 2. Reference strains demonstrating results anomalous to those published elsewhere[a]

Strain	Species and strain designation	Conventional identification	Anomaly
VH28	*A. gerencseriae* ATCC 23860	*A. georgiae*	Raffinose negative
VH36	*A. viscosus* ser. I NCTC 10951	*A. naeslundii*	Catalase negative
VH47	*A. denticolens* NCTC 11490	No identification	Trehalose positive
VH52	*A. europaeus* CCUG 32789A	No identification	Nitrate positive
VH59	*A. bernardiae* DSM 9152	No identification	β-*N*-Acetyl-glucosaminidase positive
VH60	*A. turicensis* DSM 9168	Poor differentiation	Pyrazinamidase positive
VH61	*A. radingae* DSM 9169	No identification	Esculin negative[b]
VH62	*A. phocae* DSM 10002	*A. georgiae*	Xylose positive
VH63	*A. suis* DSM 20639	*A. meyeri* or *A. naeslundii*	β-Galactosidase negative

[a] The previous publications are references 4, 5, 9, and 12.
[b] Reaction listed as positive in the previous studies but negative or slowly positive in the original description of Wust et al. (22).

RESULTS

Conventional biochemical tests. For all strains, minor amounts of acetic acid and major amounts of lactic and succinic acids were detected as products of glucose metabolism.

(i) Reference strains. The identities of 18 of the 27 strains were confirmed by conventional tests. Strains that gave anomalous results are listed in Table 2.

(ii) Clinical strains. Identifications obtained by conventional tests are listed in Table 1. Interpretation of results was sometimes difficult due to insufficient growth or the poor reproducibility of reactions. When results were inconsistent with those described for recognized species, strains are designated "most like" followed by the species name.

PMS. By PMS, strains VH20, VH21, and VH28 were contaminated and thus were excluded from analysis. The remaining strains formed four superclusters (superclusters A, B, C, and E) plus cluster with a single strain (cluster D) (Fig. 1; Table 3). The majority of strains clustered with members of the same species. However, five clinical strains identified by conventional tests as *Actinomyces meyeri* clustered closely with the type strain of *A. turicensis* and remotely from the type strain of *A. meyeri*. Also, two strains conventionally identified as *Actinomyces georgiae* clustered with four *Actinomyces gerencseriae* strains; one strain (strain VH29) was subsequently reidentified as *A. gerencseriae*; the identity of the other strain (strain VH24) remains uncertain. A reference strain of *A. israelii* (strain VH34) inexplicably clustered with this *A. gerencseriae* group. One strain (strain VH23), conventionally identified as most like *A. viscosus*, clustered with the type strain of *A. neuii* subsp. *neuii* and was subsequently reidentified as *A. neuii* subsp. *neuii* by virtue of its abilities to produce catalase, ferment mannitol, and reduce nitrate. Strains VH7, VH9, VH19, VH27, VH31, VH43, VH47, VH52, VH58, VH59, and VH61 each formed single-strain clusters.

ARDRA. All strains were cleaved by both endonucleases, yielding 6 to 10 bands by *Hae*III and 5 to 12 bands by *Hpa*II typing (Fig. 2 and 3, respectively). The resulting *Hae*III and *Hpa*II profiles were found to be highly reproducible, allowing the assignation of permanent types to each strain (Table 3). Clinical strains yielding *Hae*III and *Hpa*II profiles indistinguishable from those of a reference strain were assigned to that species. The types obtained for *A. naeslundii* reference serovars indicated subspecies variation in *Hae*III profiles and the possible species specificity of the *Hpa*II profiles. Therefore, clinical strains were assigned to species for which the *Hpa*II profile was indistinguishable from that for a reference strain and identification by conventional biochemical tests concurred. When the latter did not concur or when strains yielded distinct profiles with both enzymes, the identity was considered to be uncertain. This approach was supported by the findings obtained by conventional biochemical tests and PMS.

(i) Reference strains. By *Hae*III typing, 17 of the 22 species represented and the three serotypes of *A. naeslundii* and two of *A. viscosus* were clearly differentiated. *Actinomyces odontolyticus*, *A. meyeri*, and *A. georgiae* strains were indistinguishable

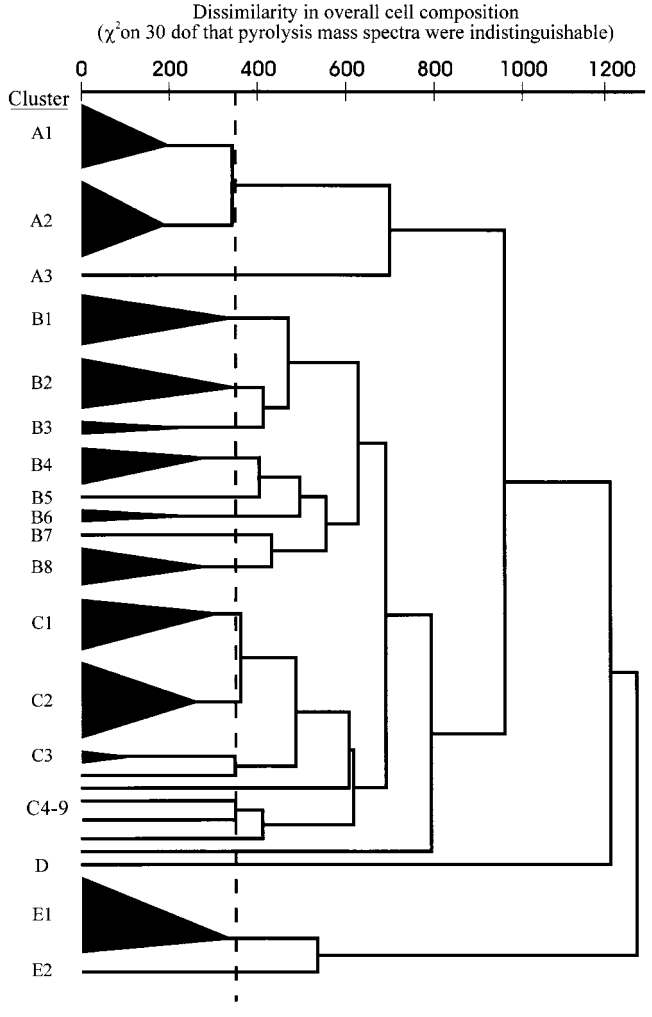

FIG. 1. Dendrogram of similarities between strains on the basis of PMS, which reflects whole-cell composition. Strain membership of the clusters is outlined in Table 3. dof, degrees of freedom.

TABLE 3. Results by ARDRA, PMS, and conventional biochemical tests, arranged by *Hpa*II profile

ARDRA profile	PMS cluster	Strain	Conventional identification	True identification
001/001	B2	VH6	*A. odontolyticus*	*A. odontolyticus* NCTC 09935
001/001	B2	VH13	*A. odontolyticus*	*A. odontolyticus*
001/001	B2	VH33	Most like *A. odontolyticus*	*A. odontolyticus*
001/001	B2	VH50	Most like *A. odontolyticus*	*A. odontolyticus*
025/001	B2	VH40	*A. odontolyticus*	*A. odontolyticus*
002/002	C9	VH7	*A. hordeovulneris*	*A. hordeovulneris* ATCC 35275
001/003	B3	VH10	*A. meyeri*	*A. meyeri* ATCC 35568
001/004	B3	VH11	*A. georgiae*	*A. georgiae* ATCC 49285
003/005	B1	VH2	*A. naeslundii*	*A. naeslundii*
003/005	C1	VH14	*A. naeslundii*	*A. naeslundii* serotype I NCTC 10301
003/005	C1	VH26	Most like *A. naeslundii*	*A. naeslundii*
007/005	B4	VH30	*A. howellii*	*A. howellii* NCTC 11636
009/005	C1	VH39	*A. naeslundii*	*A. naeslundii*
014/005	C1	VH8	*A. naeslundii*	*A. naeslundii*
014/005	B4	VH56	*A. naeslundii*	*A. naeslundii* serotype III ATCC 44340
014/005	B4	VH42	Most like *A. naeslundii*	*A. naeslundii*
017/005	C2	VH55	*A. naeslundii*	*A. naeslundii* serotype II ATCC 44339
017/005	C2	VH18	*A. viscosus*	*A. viscosus*
021/005	C2	VH54	*A. viscosus*	*A. viscosus* serotype II ATCC 27044
021/005	C2	VH17	Most like *A. viscosus*	*A. viscosus*
021/005	C2	VH46	Most like *A. viscosus*	*A. viscosus*
021/005	C2	VH49	Most like *A. viscosus*	*A. viscosus*
021/005	C1	VH3	Most like *A. viscosus*	*A. viscosus*
004/006	D	VH19	*A. pyogenes*	*A. pyogenes* NCTC 05224
005/007	B4	VH25	*A. bovis*	*A. bovis* NCTC 11535
006/008	A2	VH4	*A. gerencseriae*	*A. gerencseriae*
006/008	A2	VH16	*A. gerencseriae*	*A. gerencseriae*
006/008	A2	VH29	*A. georgiae*	*A. gerencseriae*
006/008	A2	VH32	*A. gerencseriae*	*A. gerencseriae*
006/008	A2	VH48	*A. gerencseriae*	*A. gerencseriae*
006/008	ND[a]	VH28	*A. georgiae*	*A. gerencseriae* ATCC 23860
008/009	A1	VH45	*A. israelii*	*A. israelii* ATCC 12102
008/009	A2	VH34	*A. israelii*	*A. israelii* NCTC 10236
008/009	ND	VH21	*A. israelii*	*A. israelii*
018/009	A1	VH5	*A. israelii*	*A. israelii*
018/009	ND	VH20	*A. denticolens*	*A. israelii*
020/009	A1	VH1	*A. israelii*	*A. israelii*
020/009	A1	VH15	*A. israelii*	*A. israelii*
020/009	A1	VH38	Most like *A. israelii*	*A. israelii*
009/010	B1	VH36	*A. naeslundii*	*A. viscosus* serotype I NCTC 10951
010/011	C5	VH43	*A. slackii*	*A. slackii* NCTC 11923
024/011	B7	VH31	*Actinomyces* species	Uncertain
011/012	B5	VH47	Uncertain	*A. denticolens* NCTC 11490
013/013	B1	VH51	*A. graevenitzii*	*A. graevenitzii* CCUG 27294
012/014	C6	VH52	Uncertain	*A. europaeus* CCUG 35789A
004/015	B8	VH53	*A. schaalii*	*A. schaalii* CCUG 27420
001/016	E2	VH27	Most like *A. odontolyticus*	Uncertain
015/016	E1	VH60	*A. turicensis* or *A. meyeri*	*A. turicensis* DSM 9168
015/016	E1	VH12	*A. meyeri*	*A. turicensis*
015/016	E1	VH22	*A. meyeri*	*A. turicensis*
015/016	E1	VH35	*A. meyeri*	*A. turicensis*
015/016	E1	VH41	*A. meyeri*	*A. turicensis*
015/016	E1	VH44	*A. meyeri*	*A. turicensis*
019/017	A3	VH9	*Actinomyces* species	Uncertain
022/019	C3	VH57	*A. neuii* subsp. *neuii*	*A. neuii* subsp. *neuii* DSM 8576
022/019	C3	VH23	*A. viscosus*	*A. neuii* subsp. *neuii*
022/019	C4	VH58	*A. neuii* subsp. *anitratus*	*A. neuii* subsp. *anitratus* DSM 8577
023/020	A2	VH24	Most like *A. georgiae*	Uncertain
026/021	C8	VH59	Uncertain	*A. bernardiae* DSM 9152
027/022	B6	VH37	Most like *A. pyogenes*	Uncertain
028/023	C7	VH61	Uncertain	*A. radingae* DSM 9169
029/024	B6	VH62	*A. georgiae*	*A. phocae* DSM 10002
030/025	B8	VH63	*A. meyeri* or *A. naeslundii*	*A. suis* DSM 20639

[a] ND, no data available.

(type 001), as were *A. pyogenes* and *A. schaalii* (type 004). Both *A. israelii* strains were type 008. The two *A. neuii* subspecies were type 022.

By *Hpa*II typing, 20 species were clearly differentiated; but *A. naeslundii* serotypes I, II, and III, *A. viscosus* serotype II, and *A. howellii* were indistinguishable (type 005). Both *A. israelii* strains were type 009. The two *A. neuii* subspecies were type 019. The combination of *Hae*III and *Hpa*II types allowed

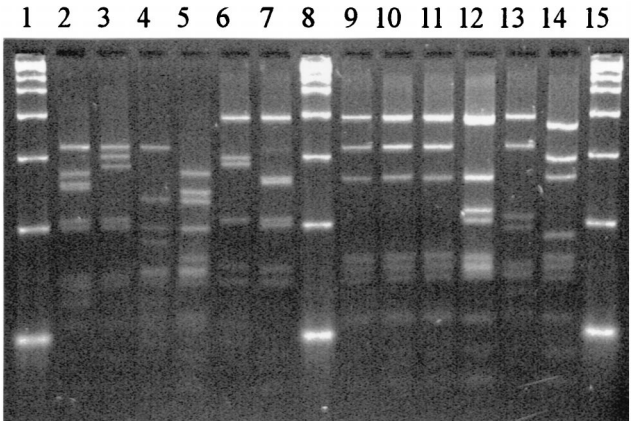

FIG. 2. *Hae*III restriction profiles for some reference strains. Lanes 1, 8, and 15, 2-kb marker; lane 2, *A. israelii* ATCC 12102; lane 3, *A. gerencseriae* ATCC 23860; lane 4, *A. naeslundii* serotype I NCTC 10301; lane 5, *A. viscosus* serotype II ATCC 27044; lane 6, *A. neuii* subsp. *neuii* DSM 8576; lane 7, *A. graevenitzii* CCUG 27294; lane 9, *A. odontolyticus* NCTC 09935; lane 10, *A. georgiae* ATCC 49285; lane 11, *A. meyeri* ATCC 35568; lane 12, *A. turicensis* DSM 9168; lane 13, *A. radingae* DSM 9169; lane 14, *A. europaeus* CCUG 32789A.

differentiation of all 22 species represented by reference strains.

(ii) Clinical strains. In reactions with both endonucleases, 16 of the 36 strains were indistinguishable from reference strains of the same species, as determined by conventional tests, and were assigned to species with confidence. Eight strains were indistinguishable by ARDRA from the reference strains of a species other than that identified by conventional tests. These comprised the five strains conventionally identified as *A. meyeri*, which were indistinguishable from *A. turicensis*; VH23, which was originally most like *A. viscosus* but which was redesignated *A. neuii* subsp. *neuii* and which gave the same profile as the *A. neuii* strains; VH29, which was originally considered to be *A. georgiae* but which was indistinguishable from *A. gerencseriae*; and VH18 (*A. viscosus*), which gave the same profile as *A. naeslundii* serotype II. These were assigned to the species determined by ARDRA, with strain VH18 being deemed a member of *A. naeslundii* genospecies 2, which includes *A. viscosus* serotype II.

Seven strains had distinct *Hae*III profiles but gave *Hpa*II profiles indistinguishable from those of reference strains of the species determined by conventional tests and were assigned to that species. One strain (strain VH31), which had a distinct *Hae*III profile and which was indistinguishable from the reference strain of *Actinomyces slackii* by *Hpa*II typing, was not clearly identified by conventional tests and was deemed to be of uncertain identity.

One strain (strain VH27) that was identified by conventional tests as most like *A. odontolyticus* gave profiles indistinguishable from those of reference strains of *A. odontolyticus* by *Hae*III typing and *A. turicensis* by *Hpa*II typing. The remaining three strains (strains VH9, VH24, and VH37) gave unique profiles in both reactions. The true identities of these four strains remain uncertain.

DISCUSSION

The application of chemotaxonomic and molecular methods has clarified the taxonomy of the genus *Actinomyces*, but the description of novel species has rendered previously published identification schemes obsolete. When few strains of a species have been studied, the reliability of identification by conventional tests is questionable. Furthermore, given the diversity within the genus, further modification of the taxonomic status of strains may be warranted.

Currently, the Public Health Laboratory Service Anaerobe Reference Unit receives over 100 referrals each year from clinical laboratories throughout England and Wales for confirmation of isolates presumptively identified as *Actinomyces* spp. There is a need for a practical and discriminatory method for the identification of clinical isolates.

The identification of *Actinomyces* to the species level by conventional biochemical tests is beset with problems. These include technical difficulties, poorly discriminatory tests, and heterogeneity within described species. In this study, nine reference strains demonstrated reactions anomalous from those published elsewhere, and this resulted in mis- or nonidentification (Table 2). Notably, *A. europaeus* was found to reduce nitrate to nitrite, and *A. bernardiae* produced β-*N*-acetyl-glucosaminidase. Anomalies, particularly in enzyme reactions, may be due to differences in test methodologies.

PMS has been shown to be highly discriminatory for a wide range of organisms but is best suited to the testing of large batches of isolates. Direct comparisons between batches are not readily made. Thus, PMS would be impractical for examination of occasional clinical isolates. However, given the low degree of confidence in results obtained by conventional tests for the identification of *Actinomyces*, evaluation of the efficacy of novel methods is difficult. In this study, PMS provided a valuable independent approach with which to compare findings. It is noteworthy and reassuring that the dendrogram derived from PMS analysis reflects the taxonomic relationships generated by 16S rDNA sequencing (4, 9, 13). *A. israelii* and *A. gerencseriae* strains formed a supercluster (supercluster A) remote from other species. Superclusters B and C were linked and contained strains identified as *A. odontolyticus*, *A. naeslundii*, *A. viscosus*, and several other species, largely represented by single reference strains. Within this group, the type strains of *A. meyeri* and *A. georgiae* formed a cluster (cluster B3) closely linked to *A. odontolyticus* strains (cluster B2). The remoteness of cluster E (*A. turicensis* strains and strain VH27) from other species raises doubts as to their place in the genus *Actinomyces*.

ARDRA has proven to be useful for discrimination of var-

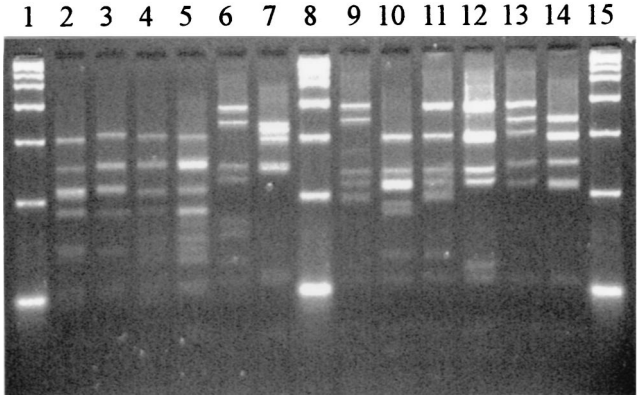

FIG. 3. *Hpa*II restriction profiles for some reference strains. Lanes 1, 8, and 15, 2-kb marker; lane 2, *A. israelii* ATCC 12102; lane 3, *A. gerencseriae* ATCC 23860; lane 4, *A. naeslundii* serotype I NCTC 10301; lane 5, *A. viscosus* serotype II ATCC 27044; lane 6, *A. neuii* subsp. *neuii* DSM 8576; lane 7, *A. graevenitzii* CCUG 27294; lane 9, *A. odontolyticus* NCTC 09935; lane 10, *A. georgiae* ATCC 49285; lane 11, *A. meyeri* ATCC 35568; lane 12, *A. turicensis* DSM 9168; lane 13, *A. radingae* DSM 9169; lane 14, *A. europaeus* CCUG 32789A.

ious bacterial species. In this study, the types generated by the combination of *Hae*III and *Hpa*II endonuclease digestion profiles correlated well with the findings obtained by PMS and conventional tests and enabled the identification of 31 of 36 clinical strains to the species level. The remaining five strains were not clearly identified by conventional tests, and three strains formed distinct clusters by PMS; the latter strains may well represent novel species or other genera.

Seven strains were identified by ARDRA and PMS as species other than those initially assigned by conventional tests. Five of these seven strains initially identified as *A. meyeri* clustered tightly with the *A. turicensis* type strain by PMS and were indistinguishable from *A. turicensis* by ARDRA. Review of conventional test results showed that the two species are poorly differentiated by the range of biochemical tests performed. The identity of one of the five strains (strain VH12) was confirmed as *A. turicensis* by 16S rDNA sequence analysis (data not shown). The strain that was apparently misidentified by PMS and ARDRA as *A. gerencseriae* had been designated *A. georgiae* by conventional tests by virtue of its inability to ferment raffinose; the morphology and other reactions were consistent with *A. gerencseriae*. This property was also demonstrated by the *A. gerencseriae* type strain; thus, raffinose fermentation appears to be an unreliable determinant in the identification of this species. The strain originally designated most like *A. viscosus* and identified as *A. neuii* by PMS and ARDRA was subsequently redesignated *A. neuii* subsp. *neuii* by virtue of catalase production, nitrate reduction, and fermentation of mannitol.

A. gerencseriae and *A. turicensis* showed homogeneous, distinct ARDRA profiles. The subdivisions observed within *A. israelii*, *A. odontolyticus*, *A. naeslundii*, and *A. viscosus* may correspond to those previously recognized by other workers (8, 17, 18). One strain of *A. viscosus* was indistinguishable from *A. naeslundii* serotype II by PMS and ARDRA, adding to the body of evidence that *A. naeslundii* and *A. viscosus* isolates form a heterogeneous complex that requires further investigation.

When species were represented by single reference strains, all strains gave distinct profiles, and none was misidentified. However, no further conclusions regarding the efficacy of ARDRA for identification of these species can be drawn without data derived from additional strains.

Current consumable costs, based on a batch of 10 isolates, were £2.80 (as of April 1999, £1 is equal to $1.60) per isolate. Each batch required approximately 3.5 h of labor spread over 1.5 days.

In conclusion, ARDRA was shown to be a simple, rapid, cost-effective, and highly discriminatory method for identification of *Actinomyces* spp. of clinical origin. Application of the method to further clinical and veterinary isolates may confirm its usefulness, and an extensive investigation of strains referred to the Anaerobe Reference Unit is in progress. Within the limitations of the current study, identification of a strain as a member of the genus *Actinomyces* by gas-liquid chromatography is a prerequisite. However, preliminary investigations of strains of *Propionibacterium* spp., *Lactobacillus* spp., and *Bifidobacterium* spp. have demonstrated the clear differentiation of strains by ARDRA (unpublished data), indicating potential for identification to the species level of members of these genera and obviating the need for gas-liquid chromatography.

Identification of clinical isolates of *Actinomyces* to the species level may be important for patient management. Additionally, a reliable identification system is essential for the discovery of the natural habitats, prevalence, and pathogenicity of recently described species. With increasing knowledge of these aspects, species-level identification of clinical isolates may become more relevant to patient management. Some species, e.g., *A. turicensis*, have been shown to be identifiable in commercial biochemical systems (15). However, genotypic methods may be advantageous in their ability to detect novel species as well as those listed in commercial databases. A practical, highly discriminatory, and cost-effective method such as ARDRA applied to many strains may greatly aid in the elucidation of the ecology and clinical spectra of *Actinomyces* species and may further clarify the taxonomy of the genus.

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