Polyacrylamide Gel Electrophoresis of Whole-Cell Preparations of *Actinomyces* spp.

SUSAN S. McCORMICK, HENRY F. MENGOLI, AND MARY A. GERENCSEK*

Department of Medical Microbiology, West Virginia University Medical Center, Morgantown, West Virginia 26506

We developed a method for polyacrylamide gel electrophoresis of whole cells of *Actinomyces* spp. The major advantage of this method is its ease of operation since it obviates the need for the preparation of bacterial extracts. Whole-cell samples were prepared by incubating washed, packed cells in 6 M urea at 37°C for 24 h. The results of disc gel electrophoresis of these whole-cell samples were compared with the results of electrophoresis of soluble protein extracts of the bacteria run under the same conditions. A large number of bands were obtained with the whole-cell preparations, and these bands were resolved better than bands obtained with the protein extracts. A total of 22 strains of *Actinomyces* spp. were examined by polyacrylamide gel electrophoresis of urea-treated whole cells. A cluster analysis of the polyacrylamide gel electrophoresis band patterns showed that the strains clustered first according to serotype and then according to species. Overall, two main divisions were identified, one containing *Actinomyces bovis*, *Actinomyces odontolyticus*, and *Actinomyces israelii* and one containing *Actinomyces viscosus* and *Actinomyces naeslundii*. This method should be a valuable tool in studying the taxonomy of *Actinomyces* spp.

In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (18), the genus *Actinomyces* is divided into five species. Since the publication of *Bergey's Manual*, the species *Corynebacterium pyogenes* has been transferred to *Actinomyces* (5), the specific epithet *Actinomyces meyeri* has been revived (3), and three new animal species, *Actinomyces denticolens* (7), *Actinomyces howellii* (8), and *Actinomyces hordeovulneris* (2), have been described. A number of problems in the classification of *Actinomyces* remain. Many normal flora isolates from oral cavities cannot be placed in a species, and several studies raise questions concerning some of the species divisions given in *Bergey's Manual*.

Two numerical taxonomy studies (10, 16) suggest that *Actinomyces israelii* may deserve recognition as a separate genus. These studies and others (6, 9) raise questions concerning the species *Actinomyces naeslundii* and *Actinomyces viscosus*. *A. naeslundii* and *A. viscosus* are closely related phenotypically, demonstrating about the same degree of relatedness in numerical studies as the two serotypes of *A. israelii* do (10). Rodent strains of *A. viscosus* are different from human isolates serologically and in some other ways. Simply combining *A. naeslundii* and *A. viscosus* into a single species does not appear to solve the problem since subclusters within each species occur in numerical studies (9, 10, 16). Deoxyribonucleic acid (DNA)-DNA homology studies support division of *A. naeslundii* and *A. viscosus* into two species and separation of rodent and human isolates of *A. viscosus* (6).

Polyacrylamide gel electrophoresis (PAGE) could prove to be valuable in answering these taxonomic questions. PAGE of soluble proteins can be used to differentiate closely related bacteria (1, 11, 13, 17) and has been used to show differences among strains of *Actinomyces* spp. (7, 8, 13). Taxonomic groupings based on PAGE of proteins closely resemble groupings based on DNA-DNA homology data (1, 15).

This study was designed to develop a simple method for PAGE analysis of *Actinomyces* spp. proteins. We devised a method in which urea-treated whole cells were used; this

method gives results comparable to those obtained with soluble protein fractions of the bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 22 strains representing five *Actinomyces* species were used in this study (Table 1). These strains were serotyped in our laboratory by using the fluorescent antibody technique. For electrophoresis, cultures were grown for 5 days at 37°C in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) containing 0.1% Tween 80. The bacteria were harvested by centrifugation and washed three times in 64 mM tris(hydroxymethyl)aminomethane buffer (pH 8.3).

Preparation of soluble protein fractions. Approximately 10 ml of washed, packed cells was mixed with 2.0 ml of glass beads (diameter, 0.1 mm) and disrupted with a Braun homogenizer (B. Braun Melsungen AG, West Germany) maintained at 4°C. The glass beads were removed by centrifugation at $1,500 \times g$ for 15 min. Cell wall debris was removed by centrifugation at $5,000 \times g$ for 30 min. The supernatant was then centrifuged at $40,000 \times g$ for 30 min, and the resulting soluble protein fraction was divided into 4.0-ml volumes and frozen at -80°C.

To prepare the samples for electrophoresis, a sample of the soluble protein was thawed, 160 μ l of a 0.9 M glacial acetic acid solution was added, and the acid-insoluble proteins were removed by centrifugation at $100 \times g$ for 10 min. The protein concentrations of the supernatants were determined by the method of Lowry et al. (12). A volume equal to 250 μ g of acid-soluble protein was combined with an equal volume of 0.5% HGT agarose (SeaKem; FMC Corp., Rockland, Maine). This stacking gel was then layered on polymerized polyacrylamide gels and allowed to harden. Two gels were prepared for each sample.

Preparation of whole-cell samples. A measured volume of washed, packed cells was mixed with an equal volume of 6.4 M urea and blended with a Vortex mixer to suspend the cells. The urea-cell mixture was incubated at 37°C for 24 h. For electrophoresis, 100 μ l of the urea-whole-cell suspension was added to an equal volume of 0.5% HGT agarose. The stacking gel mixture was layered over the polymerized

* Corresponding author.

TABLE 1. *Actinomyces* strains studied

Species	Strain (WVU no.) ^a	Serotype	Source and other designation(s) ^a
<i>A. bovis</i>	116 ^T	1	ATCC 13683 ^T ; lumpy jaw in a cow
	386	1	L. Georg; CDC strain A-1
<i>A. israelii</i>	46 ^T	1	ATCC 12102 ^T ; brain abscess
	458	1	ATCC 12836, CDC X372; human actinomycosis
	461	1	ATCC 13031, CDC A-905; bovine lumpy jaw
	307	2	ATCC 29322, P. Holm 8/46-47; human actinomycosis
<i>A. naeslundii</i>	45 ^T	1	ATCC 12104 ^T , A. Howell, Jr. strain 279 ^T ; human sinus
	777	1	S. S. Socransky strain I; dental plaque
	398A	1	WVU isolated from dental plaque
	1523	2	CDC W1544
	1602	2	CDC W752
	820	3	S. Bellack strain N16; dental plaque
	1528	3	S. Bellack strain M21b; dental plaque
<i>A. viscosus</i>	745 ^T	1	ATCC 15987 ^T , A. Howell, Jr. strain T-6 ^T ; periodontal disease in hamsters
	371	2	ATCC 19246, P. Negroni 112; human actinomycosis
	626	2	C. S. Cummins; VPI 3426; human mouth
	627	2	C. S. Cummins; VPI 3428; human mouth
	845	2	WVU isolate from dental plaque
<i>A. odontolyticus</i>	1617 ^T	1	ATCC 17929 ^T , CDC X363 ^T ; deep carious lesion
	1618	1	ATCC 17982, NCTC 9931; carious lesion of dentine
	482	2	ATCC 29323; WVU isolate from dental plaque
	750	2	L. Georg; CDC W1043; sputum

^a WVU, West Virginia University, Morgantown; CDC, Centers for Disease Control, Atlanta, Ga.; ATCC, American Type Culture Collection, Rockville, Md.; VPI, Virginia Polytechnic Institute and State University, Blacksburg.

electrophoresis gels and allowed to harden. Two gels were prepared for each strain.

Electrophoresis. Initial experiments demonstrated that, for *Actinomyces* spp., increased resolution and more bands could be obtained with acidic gels than with alkaline gels. Consequently, the polyacrylamide gel system was prepared by the method of Panyim and Chalkley (14), using 15.0% polyacrylamide gels in 6.25 M urea at a final pH of 3.2. Pararosaniline hydrochloride suspended in 20% (wt/vol) glycerol in 0.9 M glacial acetic acid was layered on top of the stacking gels so that the course of migration could be followed during electrophoresis. Each electrophoresis run included two preparations of horse cytochrome *c* (5.0% in 0.9 M glacial acetic acid) to serve as the unity value for relative migration (R_m) normalization.

A total of 18 tube gels were placed in a model 155 electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.) which was constantly cooled to 10°C by a circulating water bath (Brinkmann Instruments Inc., Long Island,

N.Y.). A glacial acetic acid (0.9 M) running buffer was used in both the upper and lower chambers. Electrophoresis was performed at a constant current of 0.44 mA/tube by using a Biochrom 2103 power supply (LKB Instruments Inc., Rockville, Md.) set at 50 W, 10V, and 8 mA until the band of cytochrome *c* was within 1.0 cm of the end of the gel.

The gels were stained for 3 days in Spencer reagent (4, 19) and then destained for 3 days in a model 172A diffusion destainer (Bio-Rad) containing 5.0% glacial acetic acid.

Data analysis. The PAGE band patterns were observed on a light table (Ednalite Corp., Peekskill, N.Y.) with a green and yellow filter combination and were drawn onto centimeter graph paper for measurements. The distances migrated by the bands were divided by the distance migrated by the cytochrome *c*, yielding R_m values. This normalization of band patterns allowed inter- and intrarun comparisons. The band patterns were then redrawn onto centimeter graph paper by using the normalized R_m values.

A composite gel pattern of all of the bands of the 22 strains

FIG. 1. Comparison of PAGE protein band patterns of soluble protein extracts (E) and whole-cell preparations (WC) of *A. bovis* WVU 116^T and *A. viscosus* WVU 626, WVU 745^T, and WVU 371. The anode was at the top.

FIG. 2. PAGE band patterns of whole-cell preparations of *A. bovis* WVU 116^T and WVU 386, *A. odontolyticus* WVU 1617^T, WVU 1618, WVU 482, and WVU 750, and *A. israelii* WVU 46^T, WVU 458, WVU 461, and WVU 307. The anode was at the top. Significant bands are indicated by arrowheads and dotted lines. The band at an R_m value of 0.70 was a common genus band; R_m value of 0.62 was an *A. bovis* species-specific band; the bands at R_m values of 0.30 and 0.42 were *A. odontolyticus* species-specific bands; and the band at an R_m value of 0.34 was an *A. israelii* species-specific band.

was drawn. The band pattern data were then reduced to a binary code, with 1 denoting the presence of a band and 0 denoting the absence of a band. Cluster analysis was performed by using the Numerical Taxonomy System of Multivariate Statistical Program network (State University of New York at Stony Brook/West Virginia University Computer Network System). This analysis incorporated a rectangular 22- by -72 matrix and the unweighted pair group method, using arithmetic averages. The 22 strains were clustered in decreasing order of similarity by using the calculated cophenetic correlation coefficients, a mathematical expression of degree of similarity.

RESULTS

The PAGE patterns obtained from the soluble protein extracts and from the urea-treated whole-cell preparations are shown in Fig. 1. Some common bands were obtained with the two systems, but a number of high-molecular-weight proteins not observed in the extract patterns appeared with the whole-cell system. Smeared bands were resolved into discrete bands when the whole-cell preparations were used. Nearly identical staining intensity of common bands was observed when 100 μ l of a whole-cell preparation or 250 μ g of soluble protein was used. We found

that the degree of breakage was difficult to control in preparing the protein extracts and that proteolysis occurred in these extracts due to bacterial enzymes. Protease inhibitors were not satisfactory in controlling this proteolysis. Storage of the extracts as frozen samples was the best method of preserving the proteins.

The most consistent electrophoresis results were obtained with the urea-treated whole cells. The reproducibility of the whole-cell PAGE system was tested by (i) using different preparations of a single strain (strain WVU 626) grown in different batches of culture medium, (ii) running identical gels in different positions in the electrophoresis chamber, (iii) incubating the cells in urea for 48 h instead of 24 h, and (iv) freezing the urea-whole-cell preparation before electrophoresis. Almost identical band patterns were obtained with the different growth preparations and with the different urea treatments, and identical band patterns were produced when samples were run in different locations in the electrophoresis chamber (data not shown).

The band patterns obtained from PAGE of whole cells of 22 *Actinomyces* spp. strains are shown in Fig. 2 and 3. One band common to the genus (R_m , 0.70) could be identified. In addition, seven species-specific bands were identified, one in *Actinomyces bovis* (R_m , 0.62), two in *Actinomyces odontolyticus* (R_m , 0.30 and 0.42), one in *A. israelii* (R_m ,

FIG. 3. PAGE band patterns of whole-cell preparations of *A. viscosus* WVU 745^T, WVU 371, WVU 626, WVU 627, and WVU 845 and *A. naeslundii* WVU 45^T, WVU 777, WVU 398A, WVU 1523, WVU 1602, WVU 820, and WVU 1528. The anode was at the top. Significant bands are indicated by arrowheads and dotted lines. The band at an R_m value of 0.70 was a common genus band; the band at an R_m value of 0.11 was an *A. viscosus* species-specific band; and the bands at R_m at values of 0.025 and 0.28 were *A. naeslundii* species-specific bands.

FIG. 4. Dendrogram showing similarity of 22 strains of *Actinomyces* spp. based on PAGE of whole-cell preparations. ST 1, Serotype 1; ST2, serotype 2; ST3, serotype 3. The abscissa indicates percentages of similarity.

0.34), one in *A. viscosus* (R_m , 0.11), and two in *A. naeslundii* (R_m , 0.025 and 0.28). In a few cases serotype-specific bands were found. For example, *A. israelii* serotype 1 (strains WVU 46^T [T = type strain], WVU 458, and WVU 461) showed three bands (R_m , 0.27, 0.38, and 0.50) not found in *A. israelii* serotype 2 strain WVU 307.

Although species differences could be identified by visually examining the protein band patterns, these differences were more evident after a computerized cluster analysis of the data. The dendrogram obtained from the cluster analysis is shown in Fig. 4. The strains clustered first according to serotype and then according to species. Finally, they clustered into two major divisions, one containing *A. bovis*, *A. odontolyticus*, and *A. israelii* and the second containing *A. viscosus* and *A. naeslundii*. *A. bovis* was more closely related to *A. odontolyticus* than either of these species was to *A. israelii*. *A. viscosus* and *A. naeslundii* grouped together, but the linkage level (73%) was less than that of the other major grouping (78%).

Since preparation of the dendrogram in Fig. 4, an additional analysis of data obtained from different preparations of some of the same strains and from some additional strains has shown that the groupings reported here are reproducible. For example, additional strains of *A. bovis* serotype 1 were identical to the two strains (strains WVU 116^T and WVU 386) previously tested. These strains showed 81% similarity with *A. odontolyticus*. Similarly, two additional strains of *A. viscosus* serotype 1 clustered with strain WVU 745^T at the 98% level, and the cluster joined the *A. viscosus* serotype 2 cluster at a similarity level of 84%.

DISCUSSION

PAGE of cellular protein preparations yields information concerning essentially the entire population of proteins of a microorganism and gives information roughly equivalent to that obtained in DNA-DNA homology studies (1, 15). Established PAGE techniques include preparation of a soluble protein fraction of the organism for electrophoresis. The technique described here eliminates this step and substitutes

incubation of washed, whole cells in 6 M urea to release cellular contents. Microscopic observations showed that urea potentiated cellular swelling. Cell contents were probably released by denaturation of structural proteins, leading to loss of integrity of the cell wall. Additionally, as urea equilibrium was reached, the osmotic concentration within the cells would have exceeded that of the medium, favoring water movement into the cells. With *Actinomyces* spp., whole-cell preparations gave more bands which were better resolved than those obtained with protein extract preparations. The major advantage of the whole-cell system is ease of operation since it eliminates the need for extract preparation.

PAGE of soluble proteins has been used previously to show similarities and differences among strains of *Actinomyces* spp. (7, 8, 13). Dent and Williams (7, 8) first recognized that the strains now classified as *A. denticolens* and *A. howellii* are different from typical *A. naeslundii* by observing band patterns after sodium dodecyl sulfate-PAGE. In the present study 22 strains representing five *Actinomyces* species were classified according to their urea-whole-cell PAGE patterns. When a cluster analysis of the band patterns was performed, the strains clustered first into serotypes and then into species as determined by other methods. Too few strains have been studied to use these data to address the classification problems in the genus, but our results indicate that PAGE of whole-cell preparations is a useful tool for classifying *Actinomyces* spp. The data available do show that the serotypes of *A. naeslundii* are less closely related than those of *A. israelii* and *A. odontolyticus*. Even the serotypes of *A. viscosus* which probably represent different species are slightly more closely related (85%) than are the serotypes of *A. naeslundii* (82 to 83%). This suggests that there are additional species among the strains now classified as *A. naeslundii*.

ACKNOWLEDGMENT

This research was supported by grant 7700143 from the West Virginia University School of Dentistry Dental Corp.

LITERATURE CITED

1. Biavati, B., V. Scardovi, and W. E. C. Moore. 1982. Electrophoretic patterns of proteins in the genus *Bifidobacterium* and proposal of four new species. *Int. J. Syst. Bacteriol.* **32**:358-373.
2. Buchanan, A. M., J. L. Scott, M. A. Gerencser, B. L. Beaman, S. Jang, and E. L. Biberstein. 1984. *Actinomyces hordeovulneris* sp. nov., an agent of canine actinomycosis. *Int. J. Syst. Bacteriol.* **34**:439-443.
3. Cato, E. P., W. E. C. Moore, G. Nygaard, and L. V. Holdeman. 1984. *Actinomyces meyeri* sp. nov., specific epithet rev. *Int. J. Syst. Bacteriol.* **34**:487-489.
4. Chrambach, A., R. A. Reisfeld, M. Wyckoff, and J. Zaccari. 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. *Anal. Biochem.* **20**:150-154.
5. Collins, M. D., and D. Jones. 1982. Reclassification of *Corynebacterium pyogenes* (Gluge) in the genus *Actinomyces*, as *Actinomyces pyogenes* comb. nov. *J. Gen. Microbiol.* **128**:901-903.
6. Coykendall, A. L., and A. J. Munzenmaier. 1979. Deoxyribonucleic acid hybridization among strains of *Actinomyces viscosus* and *Actinomyces naeslundii*. *Int. J. Syst. Bacteriol.* **29**:234-240.
7. Dent, V. E., and R. A. D. Williams. 1984. *Actinomyces denticolens* Dent and Williams sp. nov.: a new species from the dental plaque of cattle. *J. Appl. Microbiol.* **56**:183-192.
8. Dent, V. E., and R. A. D. Williams. 1984. *Actinomyces howellii*, a new species from the dental plaque of dairy cattle. *Int. J. Syst. Bacteriol.* **34**:316-320.
9. Fillery, E. D., G. H. Bowden, and J. M. Hardie. 1978. Comparison of strains of bacteria designated *Actinomyces viscosus* and *Actinomyces naeslundii*. *Caries Res.* **12**:299-312.
10. Holmberg, K., and C. E. Nord. 1975. Numerical taxonomy and laboratory identification of *Actinomyces*, and *Arachnia* and some related bacteria. *J. Gen. Microbiol.* **91**:17-44.
11. Kersters, K., and J. DeLey. 1975. Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. *J. Gen. Microbiol.* **87**:333-342.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
13. Moore, W. E. C., D. E. Hash, L. V. Holdeman, and E. P. Cato. 1980. Polyacrylamide slab gel electrophoresis of soluble proteins for studies of bacterial floras. *Appl. Environ. Microbiol.* **39**:900-907.
14. Panyim, S., and R. Chalkley. 1969. High resolution acrylamide gel electrophoresis of histones. *Arch. Biochem. Biophys.* **130**:337-346.
15. Russell, R. R. B. 1976. Classification of *Streptococcus mutans* strains by SDS gel electrophoresis. *Microbios Lett.* **2**:55-59.
16. Schofield, G. M., and K. P. Schaal. 1981. A numerical taxonomic study of members of the *Actinomycetaceae* and related taxa. *J. Gen. Microbiol.* **127**:237-259.
17. Seiter, J. A., and J. M. Jay. 1980. Application of polyacrylamide gel electrophoresis to the characterization and identification of *Arthrobacter* species. *Int. J. Syst. Bacteriol.* **30**:460-465.
18. Slack, J. M. 1974. Genus *Actinomyces*, p. 660-667. In R. E. Buchanan and N. E. Gibbons (ed.), *Bergey's manual of determinative bacteriology*, 8th ed. The Williams & Wilkins Co., Baltimore.
19. Spencer, E. M., and T. P. King. 1971. Isoelectric heterogeneity of bovine plasma albumin. *J. Biol. Chem.* **246**:201-208.