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Polyacrylamide Slab Gel Electrophoresis of Soluble Proteins for Studies of Bacterial Floras

W. E. C. MOORE,* D. E. HASH, LILLIAN V. HOLDEMAN, AND ELIZABETH P. CATO

Anaerobe Laboratory, College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

A polyacrylamide slab gel electrophoresis procedure was used to compare cellular proteins from bacterial isolates of gingival crevice floras. Isolates with identical protein patterns consistently were shown to be members of the same species. When used to screen isolates, the procedure reduced total analytical time and expense without sacrificing accuracy, and it provided additional verification of the identity of strains characterized by conventional phenotypic tests.

Several hundred proteins can be detected from individual cultures by two-dimensional electrophoretic and isoelectric focusing procedures (14), but simpler techniques yield patterns of up to 30 protein bands, which are adequate to distinguish between serotypes within genetically homologous species (8, 16, 18). Although many investigators have reported electrophoretic patterns of bacterial proteins that are distinctive for species or serotypes of bacteria (5, 10, 11, 19), there are no reports of results using the same electrophoretic procedures for determining the identity of unlabeled strains among groups representing diverse morphological or metabolic groups.

The bacterial flora of many natural habitats is exceedingly complex. A major deterrent to detailed studies of such floras is the cost of identifying sufficient isolates from each specimen to provide a reliable estimate of the types of bacteria present in the flora. Therefore, numerous investigators of the bacterial flora of various ecological sites do not attempt to determine the various species present but only report the genera or the morphotypes of the bacteria isolated. This identification provides only limited information, since species representing varied biological activities may be included in one genus or in one morphological group. We report here the results of using polyacrylamide gel electrophoresis to identify strains of the same species from such specimens.

MATERIALS AND METHODS

The gels and solutions selected were modifications of those previously used by Ames (3), Ornstein (15), Davis (4), Laemmli (12), Gordon (7), Allen (1), George and Sargent (6), Allen and Maurer (2), Gross et al. (8), and D. A. Ferguson, Jr. (personal communication). A Sigma technical bulletin (Sigma Tech. Bull. 106B, Sigma Chemical Co., St. Louis, Mo.) also was informative.

The vertical tank electrophoresis apparatus described by Studier (17) (model 100, Aquebogue Machine and Repair Shop, Aquebogue, N.Y.) was modified slightly. Two gaskets of amber latex tubing (1.6-mm bore, 0.8-mm wall) were cemented (Duco Cement) to the face of the apparatus. The upper gasket was shaped into a "U" by a wire in the bore. The lower gasket formed a convex curve above the lower buffer chamber (Fig. 1). The gaskets formed a tight seal between the gel plates and the apparatus without any grease. An LKB model 2103 power supply was used.

The cleaned glass gel plates were wiped with acetone. A 1.2-mm Lucite spacer strip was placed between and 2 mm from the bottom of the two plates. Spacer strips were placed on the two sides 4 mm from the edges at the top and 10 mm from the edges where they touched the bottom spacer strip so that the gel chamber was narrower at the bottom. The plates and strips were clamped together with a spring-type binder clip at each side and each bottom corner. The outer edges of the strips were sealed with molten 2% agar.

Protective gloves were used when acrylamide powders and unpolymerized solutions were handled (13). Resolving gels (8.5%) contained 11.3 g of acrylamide (Bio-Rad), 0.3 g of *N,N'*-methylenebisacrylamide (Bio-Rad), and 50 ml of 0.375 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (pH 8.8) (23.85 g of Tris preset pH crystals, pH 8.8 at 25°C [Sigma] in 500 ml of distilled water). The mixture was heated to dissolve the acrylamide and made up to 133 ml with pH 8.8 buffer and filtered through rapid-flow paper (Eaton-Dikeman grade 615), and 0.06 ml of TEMED (*N,N,N',N'*-tetramethylethylenediamine; Bio-Rad) was added and mixed. A 32-ml amount of the mixture was drawn into a 50-ml syringe and degassed by placing a gloved finger over the syringe tip, pulling back on the plunger to maintain a reduced pressure, and tapping the syringe vigorously against the edge of the bench. The solution was thoroughly degassed twice. A 0.3-ml portion of freshly prepared aqueous 10% (wt/vol) ammonium persulfate solution was injected through the tip of the 50-ml syringe, and the mixture was degassed once. An 18-gauge needle was affixed, and the gels were cast to a depth of 9 cm each. The syringe was rinsed with water and the process was repeated to make eight gels.

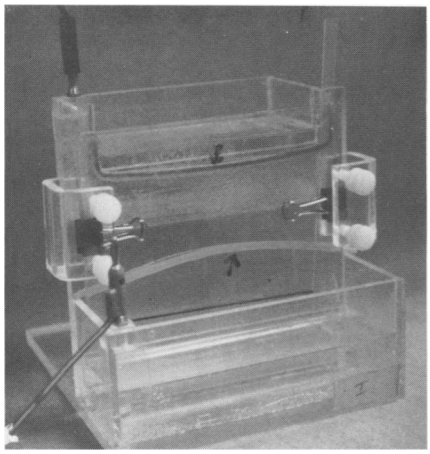

FIG. 1. *Vertical electrophoresis apparatus with gaskets cemented in place (arrows point to upper "U"-shaped gasket and lower convex gasket) and gel plates clamped to the unit.*

After the resolving gels were polymerized for 10 to 20 min, the unpolymerized acrylamide was rinsed from the surface with distilled water, the clips were removed from the bottom corners, and the bottom spacer strips were removed. The gels were placed in plastic bags with pH 8.8 buffer on top of and under the gels. The gels could be used the same day or stored at 4°C for several weeks. Before electrophoresis of samples, the pH 8.8 buffer was rinsed from stored resolving gels with distilled water, and all excess moisture was drained from the surface of the gels.

The stacking gels (4.7%) were poured from a previously prepared solution that contained 2.5 g of acrylamide and 0.07 g of *N,N'*-methylenebisacrylamide added to 15 ml of 0.15 M Tris-chloride buffer (pH 7.0) (5.82 g of Tris preset pH crystals, pH 7.0 at 25°C [Sigma] in 250 ml of distilled water). The mixture was heated to dissolve the solute, made up to 53 ml with pH 7.0 buffer, and filtered. Ten-milliliter amounts were dispensed into 12-ml screw-capped tubes, which were stored at 4°C for no more than 1 week. For use, 7 drops of tracking dye (0.25% bromophenol blue in water [wt/vol]) and 0.01 ml of TEMED were added to 10 ml of the stacking gel solution. The mixture was degassed in a 50-ml syringe, 0.1 ml of freshly prepared 10% ammonium persulfate solution was added, and the mixture was degassed once. The solution was injected over each of three resolving gels while 20-tooth combs were inserted.

After the stacking gels solidified (about 20 min), the combs were removed, the plates were clamped to the apparatus (Fig. 1), and 0.025 M Tris-0.2 M glycine electrode buffer (5.4 g of Tris [Bio-Rad]-27.0 g of glycine [Bio-Rad], 1,800 ml of distilled water) was added to the reservoirs. Each well was rinsed by withdrawing and discarding some electrode buffer with a syringe and a 21-gauge needle. Distorted wells were straightened with the needle. Trapped air was forced from the groove beneath the resolving gel with electrode buffer from a syringe and bent needle.

Samples were prepared from 5 ml of prereduced anaerobically sterilized BHIC or BHIC-Tween cultures (supplemented brain heart infusion broth [9] with 0.1% calcium carbonate [BHIC] with 0.025% Tween-80). The bacterial cells were harvested in plastic 15-ml conical centrifuge tubes in an angle-head centrifuge at 8,000 × *g* for 10 min. The supernatant was decanted and drained from the cell pellet. (The supernatant was acidified and used for analysis of the fermentation products by chromatography [9].) Approximately 0.15 g of 74- to 110-μm-diameter glass beads (class IV, no. 1420, type C; Cataphote Division of Ferro Corp. Jackson, Miss.), measured with a dipper, and 0.15 ml of pH 7.0 Tris-chloride buffer were added to the unwashed cell pellet. These samples could be stored at −20°C and then thawed before the cells were broken. If the samples were analyzed within 1 h, the cells were broken without freezing.

The bacterial cells were broken by holding the tube either against a Vortex mixer for 4 min or in an apparatus (Fig. 2) in which 20 tubes could be processed at one time. The bottoms of the tubes, immersed in an ice bath, moved a distance of 5 mm at a rate of 1,800 times per min for two 2-min periods. The samples were kept chilled. One-third volume of powdered sucrose per volume of sample was then added to saturate the samples. For gram-negative species, the cells were broken as described above, but the cellular debris was packed by centrifugation at 8,000 × *g* for 10 min before the sucrose was added to the supernatant.

Samples (30 μl) were layered under the buffer solution in the wells. A reference culture sample was run on each gel. Electrophoresis was run at room temperature at 150 V (constant) and about 33 mA initial current. Electrophoresis was stopped when the tracking dye reached the bottom of the gel (in about 2.5 h, depending on the room temperature).

To stain the gels, each was removed from the plates and placed in 12% trichloroacetic acid (454 g of trichloroacetic acid-3,624 ml of distilled water) for 20 min at room temperature. The trichloroacetic acid was replaced with 0.08% Coomassie blue stain (4.0 g of Brilliant Blue R, 60% dye content [Sigma], 900 ml of

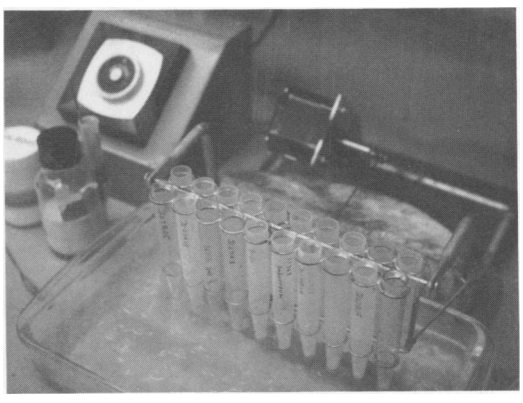

FIG. 2. *Apparatus for breaking 20 samples of bacterial cells. The 1,800-rpm motor and swinging 20 tube rack are on a weighted base. An eccentric drive shaft is off-set 2.5 mm to drive the pitman arm a distance of 5 mm per revolution.*

methanol, 400 ml of glacial acetic acid, and 1,800 ml of distilled water) for 10 min. The gels were destained with 10% glacial acetic acid in water (vol/vol). The trichloroacetic acid and the stain solution were saved and re-used.

After 1 or 2 h at room temperature, the depth of color was adjusted by adding either small amounts of stain or fresh destain solution. After 4 h or more the gels were transferred to plastic "ziploc" bags for photographing and storage and the excess destain solution was discarded.

The gels were photographed on a glow box (Instruments for Research and Industry, Cheltenham, Pa.) with a Calumet camera (4 by 5 in. [ca. 10.1 by 14.6 cm]), a 135-mm Caltar SII f/5.6 lens, a yellow filter, and a Polaroid filmpack holder. We used Polaroid 107 film and a lens setting of 1/125 s at f/11-22. Both the stored gels and the pictures were useful for visual comparisons of the protein band patterns.

RESULTS

Analysis of results. Example protein electrophoretic patterns of isolates from the human gingival sulcus are shown in Fig. 3-5. The isolates were obtained by randomized selection of colonies and grouped according to their cellular morphology and Gram stain characteristics before electrophoretic analysis. The species identifications were made subsequently by our conventional phenotypic tests (9) with additional standard tests for streptococci and fluorescent antibody tests for *Actinomyces* sp.

With some strains or species, a definitive pattern was not obtained: cell or protein yield was insufficient, or other cellular components interfered. A minimum of three distinct bands was required for reliable comparisons of strains. Only patterns from the same morphological groups of bacteria were compared. As described by others (8), several distinct and reproducible patterns were produced by different strains of some species, but only one pattern was seen among many strains of some other species.

To determine whether or not two patterns were identical, it was helpful to compare the original gels and to take two photographs of each gel, mark the reference protein (the heavy band of *Streptococcus faecalis* strain U4-20) with a pen line across one photograph, and cut off each lane in sequence so that the patterns could each be placed directly adjacent to every other pattern.

Photographs of patterns from reference strains and strains that were fully characterized were taped to clear plastic cards. These were used to verify conventional identifications of later isolates. The patterns were not used for identification without conventional characterizations, although it was often possible to predict

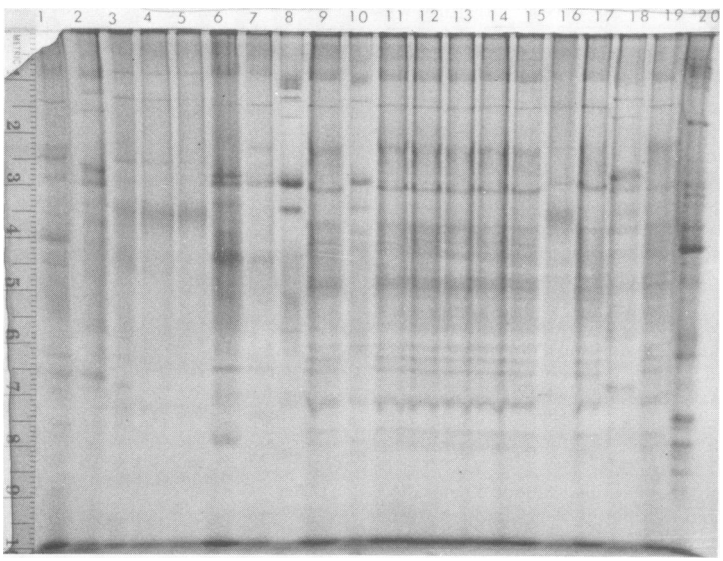

FIG. 3. *Patterns from 19 isolates, morphologically resembling Actinomyces, from gingival crevice samples. The identifications were based on phenotypic characteristics and fluorescent antibody (FA) reactions. However, the FA tests were not made on identical pattern isolates in this set because the supply of FA conjugate was limited. The patterns that were considered to be identical are listed together within parentheses. Fluorescent antibody reactions are given in brackets. Lane (1) A. viscosus [+, cross-reacting with A. naeslundii conjugate]; (2) A. meyeri [−]; (3) A. israellii [−]; (4, 5) A. israelii I [+]; (6) (18) A. naeslundii [−]; (7) actinomyces serotype WV963 [+]; (8) (10) facultative gram-positive rod D-5 [−]; (9, 11, 12, 13, 14, 15, 17, 19) A. naeslundii [+]; (16) actinomyces phenotypically similar to WV963 [−]; (20) S. faecalis reference strain U4-20.*

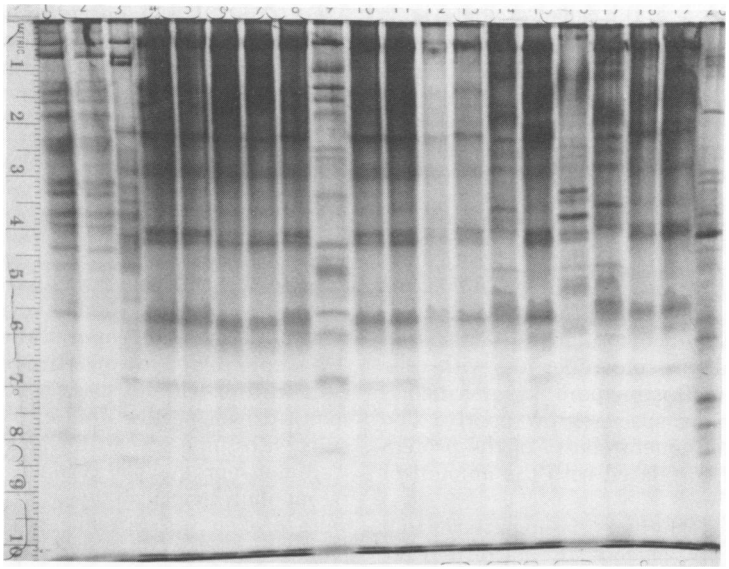

FIG. 4. *Patterns of 19 isolates of gram-negative rods from gingival crevice samples. The patterns that were considered identical are listed together within parentheses. The given identifications were based on phenotypic characteristics. Lane (1, 2) Capnocytophaga gingivalis; (3) Bacteroides bivius; (4, 5, 6, 7, 8, 10, 11, 12, 13, 18, 19) (14, 17) (15) Fusobacterium nucleatum—different pattern groups; (9) Leptotrichia buccalis; (16) Capnocytophaga ochracea; (20) S. faecalis reference strain U4-20.*

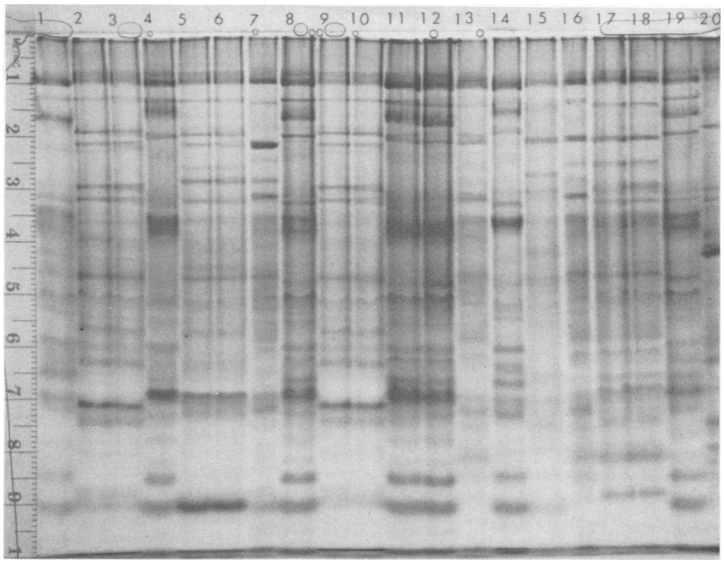

FIG. 5. *Patterns from 19 isolates of gram-positive cocci and a control strain of S. faecalis. The given identifications were based on phenotypic characteristics. The patterns that were considered to be identical are listed together within parentheses. Lane (1) (4, 8, 11, 12, 19) (14) S. sanguis type II—different pattern groups; (2, 3, 9, 10) (5, 6) Streptococcus species D-23—different pattern groups of this undescribed species; (7) S. sobrinus; (13, 16) Streptococcus species D-16—undescribed species; (15) (17, 18) S. sanguis type I; (20) S. faecalis reference strain U4-20.*

correctly the identity of some species on the basis of tests for Gram reaction, aerotolerance, fermentation acids, and the electrophoretic pattern of soluble proteins.

Effect of media. Cellular debris had little or no effect on the clarity of the protein patterns from gram-positive species grown in a low-carbohydrate medium, but a darkly stained back-

ground was produced if the cells were harvested from a medium rich in glucose (Fig. 6) or other fermentable carbohydrate. The smeared background was not eliminated when smaller samples were used.

The cellular debris of gram-negative cells often caused a dark-staining smeared background. The patterns were clearer if the debris was removed by centrifugation, but with some species the background was not eliminated by washing the cell pellet before the cells were broken, or by growing the cells in peptone-yeast extract (PY; 9), or BHI (which contains 0.2% glucose). The background was sometimes less with cells grown in PY, but this medium was unsatisfactory because many species grow poorly. The intensity and consequent clarity of the protein bands generally correlated with the amount of growth.

In some species that had protein patterns that were unaffected by glucose, the patterns were quite similar with cells from different growth media whether or not the different carbohydrates were fermented. Typical effects of different media are shown in Fig. 7.

Chopped-meat broth contained protein that interfered with the electrophoretic analysis. It was important to exclude meat particles if the cultures to be analyzed were inoculated from chopped-meat cultures.

To standardize the patterns as much as possible, most species were harvested from either supplemented BHIC for gram-negative species or supplemented BHIC-Tween for most gram-positive isolates. Exceptions were made for fastidious species that would grow only in PY-arginine (9) or that required an additional nutrient. Resulting patterns were comparable among strains within species when they were grown in the same medium. If serum was required for growth, the cell pellet was washed before the cells were broken to remove serum proteins which otherwise appeared in the patterns.

Sample preparation. Grinding the bacterial cells with or without glass beads in tubes with motor-driven pestles produced satisfactory results with some species but not others. Results were not reproducible within strains among different morphological types of cells when cells were broken with a sonic probe. *Bacteroides fragilis* proteins were relatively stable but *Propionibacterium acnes* proteins appeared to be sensitive to heat generated during disruption by any treatment.

The most convenient and reliable procedure for breaking most bacterial species was by agitating the cells with 74- to 110-μm glass beads in conical centrifuge tubes. Smaller or larger beads or sand or pumice particles were less effective. Conical tubes with narrow tips (e.g., Bel-Art

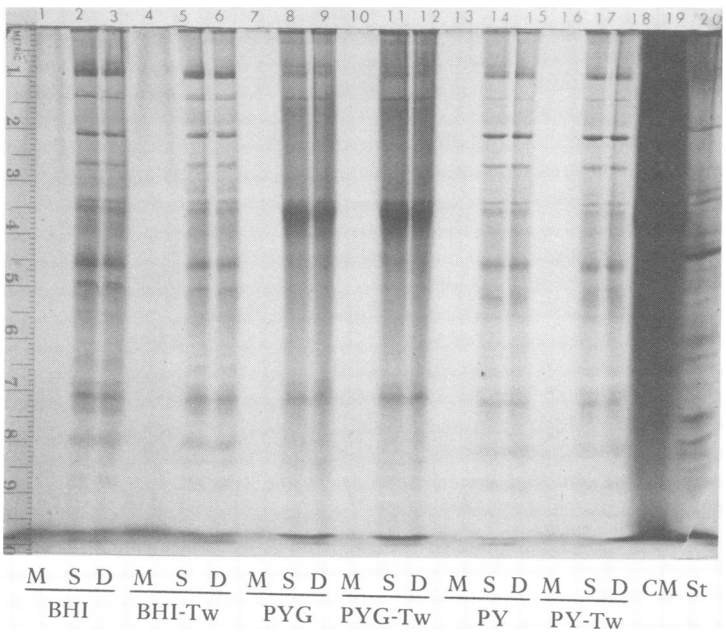

FIG. 6. *Electrophoretic protein patterns from 30-μl samples of media and from bacterial cell preparations of Streptococcus species D-16. M, Uninoculated medium. (Lane 1) BHI; (4) BHI-Tween; (7) PYG (peptone-yeast extract [PY]) with 1% glucose, (10) PYG-Tween; (13) PY; (16) PY-Tween; (19) CM (chopped-meat broth). S, Supernatant of preparation of broken cells grown in the medium in the preceding lane. D, Cellular debris from the preparation used in the preceding lane. Lane 20, St, Reference sample of S. faecalis strain U4-20.*

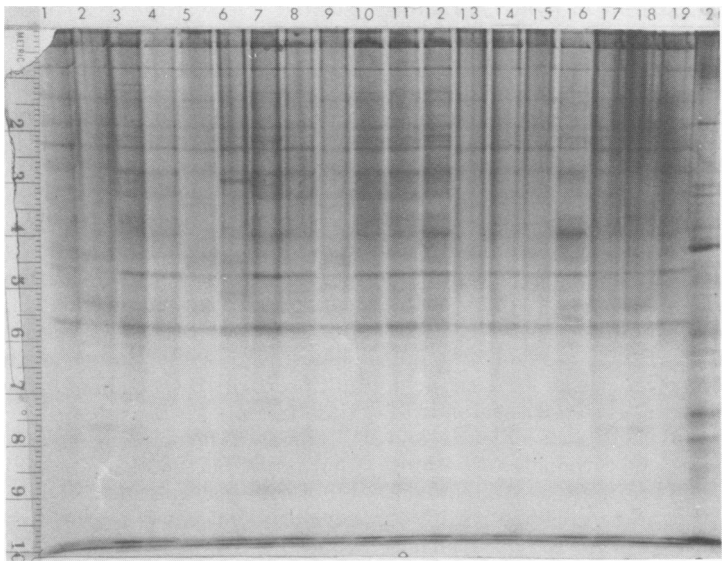

FIG. 7. *Electrophoretic pattern of protein bands from Bacteroides ovatus strain U20-1 grown in various carbohydrates in PY base. The media were as follows: (1) PY; (2) amygdalin; (3) arabinose; (4) cellobiose; (5) erythritol; (6) fructose; (7) glucose; (8) glycogen; (9) inositol; (10) lactose; (11) maltose; (12) mannose; (13) melezitose; (14) melibiose; (15) raffinose; (16) rhamnose; (17) ribose; (18) salicin; (19) sucrose. Lane 20, S. faecalis reference strain U4-20 from BHI-Tween.*

plastic 15-ml centrifuge tubes) were less effective than tubes with blunt tips (e.g., Nalgene). As with the other methods, more vigorous or prolonged treatment decreased the yield of proteins from some species. The conditions for operation of the 20-tube cell breaker that we designed were determined after tests with variable speed motors and a series of drive shafts with varying amounts of eccentric off-set.

The concentration of protein in samples from some species decreased rapidly after the cells were broken. Protease inhibitors failed to protect the proteins, but chilling during and after breaking the cells preserved proteins of many species and produced clearer protein patterns.

Between pH 4.0 and 12.8, the buffer in which the cells of different species were broken had little or no effect on the amount of protein released or the migration of the proteins. To avoid other possible interactions, we used buffer similar to that in the stacking gel.

Some investigators have used sodium dodecyl sulfate (SDS) to increase the recovery of protein from cells or to modify the charge on the proteins so that their migration would more nearly correspond to their molecular sizes (3, 8). SDS also disassociates some proteins and therefore increases the number of distinct bands in the gels. When samples from cells broken in the presence of SDS were used with gels and electrode buffer containing no SDS, they produced no more bands and no more total protein than cells broken without SDS. With some species, little or no pattern was obtained when SDS was added, but clear patterns were produced in its absence. With some other species the addition of SDS to the sample buffer caused the pattern to be smeared. With still other species no difference was observed.

The addition of SDS to the gels and electrode buffer increased the number of bands. When samples from cells broken in the absence of SDS were used with gels and electrode buffer containing SDS, they produced as many bands as cells broken in the presence of SDS. Apparently the major effect of SDS occurred in the gels during electrophoresis. These conditions were tested to determine whether or not strains of one species that had different patterns would then produce patterns that were more similar. Representatives of one such multipattern species that produced reasonably clear patterns with SDS in the sample buffer were tested with and without SDS in the gels and electrode buffer. The patterns were no more similar among strains when SDS was added than when SDS was not added. The increased number of bands made visual comparisons of the patterns more difficult.

Initially we used spacer gel blanks when the resolving gels were poured. This was discontinued because, as described by Ames (3), the minor irregularities or tags of resolving gel that were produced at the stacking gel-resolving gel inter-

face caused narrow streaks of light- and dark-staining background. This observation and the dark background from gram-positive cells cultured on glucose suggested that a protein complex or agglomerate with other cellular constituents was produced at the gel interfaces. The possibility that protein elution from a complex was responsible for the dark-smeared background, produced with many species, led us to test other additives in the sample buffer in an effort to eliminate the dark-staining background and to improve the recovery or stability of the proteins. Chloroform, ether, phenol, glycerol, mercaptoethanol, methanol, ethanol, butanol, ethylenediaminetetraacetic acid, fumaric acid, toluene, xylene, benzene, Tween-80, acetonitrile, hexane, heptane, and methylene chloride and the chlorides, sulfates, and carbonates of barium, magnesium, zinc, and iron were tested. None of these additives was consistently effective.

Operating conditions. The most critical factor affecting reproducibility of the gel pattern appeared to be the sensitivity of the acrylamide solutions to oxygen. Acrylamide solutions are oxygenated during preparation and filtration. It is necessary to degas the acrylamide solutions thoroughly before the ammonium persulfate is added. We were unable to store solutions satisfactorily, even in bottles that had been flushed with nitrogen. After storage for only 1 week, the migration of reference proteins increased by 4 mm or more. If the solutions were made oxygen-free by storing iron nails in the bottles, the gels solidified before they could be poured when ammonium persulfate was added. Fortunately, the polymerized resolving gels, if kept moist, were more stable than the solutions.

Although several references suggested that at lower temperatures more current could be used to increase the migration rate of the proteins without overheating the gels, we found that the patterns were distorted as the voltage was increased unless the ionic strength of the electrode buffer was decreased. When the ionic strength was decreased, the migration rate of the protein decreased. If the gel and electrode buffers were of suitable strength for use with a given voltage, then the migration rate of the proteins increased as room temperature increased. At room temperature and 150 V, when the solute concentration in the electrode buffer was increased by half, the migration rate of all bands increased by 11%, the initial current increased to 40 mA, and the plates became warm. There was little change in the resulting patterns, but they often slanted upwards at the edges. When the concentration of solutes in the electrode buffer was cut in half, the migration rate decreased by 18% and the protein bands were poorly defined and often slanted downwards at the edges. The amount of protein in the samples also appeared to affect the distortion of the patterns at the edges of the gels.

When the pH of the resolving gel was increased from 8.8 to 9.0, the tracking dye reached the bottom of the gel in 88% of the time it took at the lower pH. But the protein bands, unexpectedly, did not move any faster and therefore moved only 88% as far as those on the control gel. Although the same relative pattern of bands could be recognized on each gel, resolution was better at pH 8.8. From these observations it appeared that the charge on the proteins was not primarily responsible for their migration in this system.

There was no one concentration of acrylamide that produced the most distinctive patterns for all of the species tested. Predictably, each protein band migrated farther as the acrylamide concentration was decreased, and some bands of larger (slower-moving) proteins were separated into two or more bands. With 9 and 10% acrylamide, the lower half of the gels often had no bands. At 7 and even 8% acrylamide, much of the protein moved with the front. For the species tested, the best distribution of bands throughout the entire gel was obtained with 8.5% acrylamide.

DISCUSSION

Among over 1,800 strains tested there were over 90 1-mm positions in which protein bands occurred. Bands less than 1 mm apart could be distinguished readily. Individual bands often had different relative densities or other distinctive characteristics, such as sharp or tailing edges. The pattern of bands was analogous to the results from a set of 90 independent phenotypic tests and was probably more reliable because it was not subject to as many errors from inoculating, analyzing, or recording results of tests from many tubes of media. Also, cultures that produce few or no positive cultural test results often produced distinctive protein band patterns.

For several months we fully characterized at least two isolates of each identical pattern group. Conventional identification of over 200 such pairs of isolates demonstrated that, without exception, each isolate with an "identical" pattern was the same species. These results indicated that it was unnecessary to further characterize more than one strain among those that were similar morphologically, produced the same acids, and had identical protein electrophoretic patterns.

It was necessary to fully characterize all isolates that had similar patterns but which differed

by one or more bands. Although most strains with similar patterns proved to be the same species, they were sometimes different species or even members of different genera.

Two people could prepare gels and analyze 114 pure cultures a day (plus a reference sample on each of six gels). Among 30 isolates from single oral specimens an average of 12 produced patterns that were identical to one or another of the other 18 isolates. Consequently, the electrophoretic analysis eliminated the need for complete characterization of about 45 of 114 isolates tested in 1 day. The cost of salary and materials for the electrophoretic analysis of 114 isolates was about 10% of the cost for complete characterization of the 45 identical isolates, so the total cost was reduced by about one-third. The two-thirds that had to be completely characterized included those that produced unacceptable patterns with too little protein to obtain visible bands, too few bands, patterns obscured by heavy backgrounds, or unique patterns.

Distinctive patterns were obtained, by the same procedure, with diverse kinds of bacteria: aerobes and anaerobes, gram-positive and gram-negative strains, rods and cocci, and fermentative and non-fermentative bacteria. When used to screen for identical isolates before identifications were made by conventional analyses, electrophoretic analysis provided greater accuracy at a much lower analytical cost. From our observations it appears that such analyses have potential application in a wide range of ecological and epidemiological studies, particularly when it is desirable to identify multiple isolates from a single specimen.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Since this manuscript was prepared, we have found that much background material can be eliminated from patterns of gram-negative bacteria by heating the broken-cell preparation at 55°C for 5 min before centrifugation.

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