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Rapid Method for Characterization of Actinomycetes by Cell Wall Composition

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To achieve a rapid identification of the cell wall components of actinomycetes, several modifications of the procedure of Cummins and Harris were developed. Purified cell walls were prepared by extraction of whole cells with 0.1 N NaOH. A 2-hr 2 N HCl hydrolysate was prepared for identification of sugars. A 2-hr 6 N HCl hydrolysate was prepared for amino acid analysis. Two-dimensional paper chromatography was run on 19-cm papers. Total time required for a single analysis was approximately 24 hr. This procedure gave qualitative results which were completely satisfactory for differentiation of certain species in the genus *Actinomyces* and in related genera.

Various criteria have been used for taxonomic separation of groups of microorganisms. Cell wall composition determinations have demonstrated important differences not revealed by conventional bacteriological techniques. Such procedures of cell wall analyses, in particular, have clearly defined members of the genus *Actinomyces*.

In their cell wall analyses, Cummins and Harris (7) determined the composition of the walls of various gram-positive organisms. A clear distinction was made between the species *A. bovis* and *A. israelii*. Subsequently, *A. naeslundii* was differentiated from these two species by its cell wall composition (13; Cummins, *personal communications*). Differentiation by the classical methods entailing observation of colony morphology and certain physiological characteristics proved to be unsatisfactory in certain cases (13). It would be extremely valuable, therefore, to have a simple and rapid cell wall analytical procedure for the identification of the various species of *Actinomyces* and closely related organisms.

Becker et al. (1) reported a method for rapid differentiation of *Norcardia* and *Streptomyces*, in which whole cells were hydrolyzed, and one-dimensional decending paper chromatography was used to determine differences in diaminopimelic acids. They did not report analyses for any other amino acids or carbohydrates.

The usual two-dimensional paper chromatographic method of determining the composition of amino acids and sugars of cell walls of microorganisms, as described by Cummins and Harris (6), requires several steps, including breakage of the cells by mechanical means, digestion with proteolytic enzymes, acid hydrolysis with 2 N H_2SO_4 for sugars and 6 N HCl for amino acids, and neutralization procedures for removal of these acids preceding the actual chromatographic procedures.

This report describes a rapid chromatographic procedure developed for routine use in the laboratory for the identification of species of *Actinomyces*. The procedure may be completed in 24 hr with cells obtained from 10 to 20 ml of broth culture; further, it has been shown to be applicable to other bacteria, particularly to gram-positive organisms.

MATERIALS AND METHODS

Microorganisms used. The organisms used are listed in Table 1.

Preparation of cells. All organisms used, except *Staphylococcus aureus* and *Streptococcus pyogenes*, were grown in the *Actinomyces* maintenance broth described by Georg, Robertstad, and Brinkman (8) at 37 C under pyrogallol-carbonate seal as described by Howell and Pine (10). *S. aureus*, Cowan I, was grown in Trypticase Soy Broth and *S. pyogenes* (group A) was grown in Todd-Hewitt Broth at 37 C.

All strains were grown in 10- to 20-ml amounts in 18-mm test tubes, and the cells were harvested at maximal growths (as determined by gross observation) after an incubation period varying from 12 hr to 5 days. The cells were collected by centrifugation and washed three times in distilled water. The cells were extracted for 1 hr with 0.5 ml of 0.1 N NaOH in a tightly sealed 10.0 screw-cap tube placed in a boiling-water bath. During this period of extraction, the optical density of the supernatant fluid at 280 mμ rose to a maximum and did not increase with further digestion of the cells. The mixture was centrifuged,

TABLE 1. *Cell wall composition of Actinomyces species and other gram-positive organisms*

Name of strain[a]	Sugars	Amino acids	Hexosamine	Muramic acid	Results of other investigations		
					Sugars	Amino acids	Reference
Actinomyces israelii 296[b] ATCC-10049	Glucose Galactose Rhamnose	Aspartic acid Glutamic acid Alanine Lysine-ornithine Comb. 4[c]	+		Glucose Galactose Rhamnose	Aspartic acid Glutamic acid Alanine Lysine-ornithine	(13)
A. naeslundii W-826 ATCC-12104	Glucose Rhamnose	Glutamic acid Alanine Lysine-ornithine Comb. 4	+		Glucose Galactose Rhamnose	Glutamic acid Alanine Lysine-ornithine	(14)
A. israelii 895 CDC-W724	Galactose	Glutamic acid Alanine Lysine-ornithine	+		Galactose	Glutamic acid Alanine Lysine-ornithine	(14)
A. israelii A601 ATCC-19038	Galactose	Glutamic acid Alanine Lysine-ornithine	+		Galactose	Glutamic acid Alanine Lysine-ornithine	(14)
A. bovis P2S ATCC-13684	Glucose Rhamnose 2-Deoxytalose	Aspartic acid Glutamic acid Alanine Lysine-ornithine Comb. 4	+		Glucose Mannose Rhamnose 2-Deoxytalose Fucose	Aspartic acid Glutamic acid Alanine Lysine-ornithine	(13)
A. eriksonii ATCC-15423 CDC-W829	Galactose	Aspartic acid Glutamic acid Alanine Lysine-ornithine	+	±	Galactose	Aspartic acid Glutamic acid Alanine Lysine	(8)
A. propionicus 699 ATCC-14157	Glucose Galactose	Aspartic acid Glutamic acid Alanine Glycine Comb. 4 Diaminopimelic acid	+		Glucose Galactose	Aspartic acid Glutamic acid Alanine Diaminopimelic acid	(4)
A. odontolyticus ATCC-17929	Glucose Galactose Mannose	Glutamic acid Alanine Lysine-ornithine Comb. 4	+		—	—	—[d]
Streptococcus pyogenes group A, type I	Rhamnose	Glutamic acid Alanine Lysine-ornithine Comb. 4	+	±	Rhamnose	Glutamic acid Alanine Lysine Glycine	(11)

Corynebacterium acnes CDC-W832	Galactose Arabinose	Glutamic acid Alanine Diaminopimelic acid	+		Galactose Arabinose	Glutamic acid Alanine Diaminopimelic acid	(14)
Streptomyces griseus CDC-B777	No sugars	Glutamic acid Alanine Glycine Diaminopimelic acid	+	±	Trace-glucose Galactose	Glutamic acid Alanine Glycine Diaminopimelic acid	(2) (15) (14)
Rothia dentocariosus ATCC-17931	Galactose	Glutamic acid Alanine Lysine-ornithine Comb. 4	+	±	Galactose	Glutamic acid Alanine Lysine-ornithine	
Bacterionema matruchotii CDC-868	Glucose Galactose Mannose	Glutamic acid Alanine Comb. 4 Diaminopimelic acid	+		Glucose Galactose	Glutamic acid Alanine Diaminopimelic acid	(14)
Staphylococcus aureus	No sugars	Glutamic acid Alanine Lysine-ornithine Glycine Comb. 4	+	+	No sugars	Glutamic acid Alanine Lysine Glycine	(6)

[a] ATCC = American Type Culture Collection; CDC = Communicable Disease Center.

[b] Although originally classified as a strain of *A. naeslundii*, recent studies on the cell wall composition (13) and studies with fluorescein-labeled globulin (12) showed that this strain was *A. israelii*.

[c] Combination of leucine, isoleucine, phenylalanine, and methionine.

[d] No comparative data.

and the cell wall deposit was washed four times in distilled water with centrifugation at 3,000 rev/min. The alkaline extract was discarded.

Hydrolysis and chromatography. The cell walls were suspended in a minimal amount of distilled water. Two-thirds of the residue of the alkaline extraction was used for detection of sugars and the remaining one-third was used for detection of amino acids.

Sugars. Samples were made to 1.0 ml in 2 N HCl in tightly sealed 10.0-ml screw cap tubes and were placed in a boiling-water bath for 2 hr. The liquid hydrolysates were transferred to small beakers. Samples, placed in a hot-water bath, were evaporated to dryness by a gentle air jet. Water (1 to 2 ml) was added, and the samples were redried three additional times to remove the HCl. Samples were redissolved in 0.3 ml of water for chromatography.

Amino acids. Samples were hydrolyzed in 1.0 ml of 6 N HCl in 10.0 ml screw-cap tubes for 2 hr. They were then dried as described above.

Chromatography. Amounts of 5 to 10 μliters of each sample were placed 20 mm from the corner of a 190-mm square of Whatman no. 1 chromatography paper. Edges of the paper were opposed and were stapled at the top, middle, and bottom to form a hollow cylinder without the edges touching.

The primary solvent used was phenol-water for both amino acids and carbohydrates. This was made by diluting 90% liquid phenol (chromatography grade) with water (80:20, v/v). The solvent vessel was either a battery jar (15 cm in diameter and 20 cm high) or a 1 gal, wide-mouth "pickle jar." Tops were well greased with vacuum grease and covered with glass plates. Migration time was 4 hr at 37 C or up to 5.5 hr at room temperature. The higher temperature provided an advantage of speed with no observable sacrifice of resolution.

Amino acid separations were conducted in an ammonia atmosphere. A 10- to 15-ml amount of a 0.3% solution of concentrated NH_4OH reagent was placed in a 25-ml beaker, which was positioned inside the paper cylinder at the bottom of the jar.

After primary migration, papers were unstapled and dried, either at 60 C for 2 hr or overnight in a chemical hood. Complete evaporation of phenol from the paper was necessary, especially for carbohydrate determinations. Three diethyl-ether washes, with fresh ether for each wash, were used on the carbohydrate papers when rapid drying was employed, but the overnight drying procedure for the removal of phenol was preferred.

For amino acids, the second-dimension solvent was 2,6-lutidine (practical grade, 95%) and water (65:35, v/v). The papers were formed into cylinders at 90° from the original stapling. Migration time and temperature were similar to that of the primary solvent.

For carbohydrates, the second-dimension solvent was butanol, pyridine, water (60:40:30, v/v). Migration time and temperature were as previously described; 37 C provided an optimal 3.5-hr migration.

Amino acids were detected by dipping the papers in a solution of 0.25% ninhydrin in 95% ethyl alcohol, drying them, and then heating them at 65 C for 2 to 5 min. All amino acids in the hydrolysate gave purple spots with this reagent, with the exception of aspartic acid and glycine. Aspartic acid gave a light blue color, and glycine gave a pink color. After identification of amino acids, the papers were dipped in a 5.0% nickel sulfate solution to preserve the chromatogram. This treatment also emphasized the primary ninhydrin spots by clearing the background and blanching many of the secondary spots. Carbohydrates were detected by dipping dried chromatograms in a solution containing 0.1 ml of 50% aqueous silver nitrate in 25 ml of acetone. The papers were dried at room temperature, and dipped into 0.5 N KOH in 95% ethyl alcohol. The papers were dried again for 2 to 5 min until dark brown spots appeared. They were then dipped into 1.0% sodium thiosulfate to remove dark background. For additional confirmation of the presence of diaminopimelic acid, one-dimensional ascending cylindrical chromatography with the solvent described by Hoare and Work (9) was carried out for 3 hr at 37 C. The spots were developed by the same procedure used for other amino acids, except that they were heated at 100 C for 2 min. Diaminopimelic acid spots were olive green, fading to yellow. Other amino acids in this solvent separation gave purple spots, and most had a higher R_F value than the slow-moving diaminopimelic acid. The isomeric form of diaminopimelic acid was not determined.

RESULTS AND DISCUSSION

The results obtained by use of the proposed method of chromatographic separation of amino acids and sugars from cell wall hydrolysates (Table 1) compared favorably with the results obtained by other investigators using the classical Cummins and Harris procedure. Typical chromatograms obtained for a strain of *A. bovis* are given in Fig. 1 and 2. Moreover, the use of this new method offered a distinct advantage in its simplicity and its obvious time-saving devices. The results were reproduced with several cultures of the same strain grown at different times. No significant qualitative variations were observed in the amino acid and sugar patterns (Fig. 1 and 2). As stated by Cummins and Harris (6), some cell walls hydrolyzed less than 24 hr produced an unknown spot that corresponded to glycine when run in phenol-water plus NH_3 and lutidine-water. This same spot was encountered in some of our hydrolysates, but it was not significant enough to cause concern. Cummins (5) and Cummins and Harris (7) reported that the alkaline extraction of all cell walls gave a smaller number of amino acids on chromatography than those obtained after cell disintegration and enzymatic digestion. In contrast, our results with alkaline extracted walls were qualitatively comparable with those obtained by the more extensive method. Some sugar spots did not appear as

large as with the conventional method, but the pattern was not altered. In an ammonia atmosphere, the lysine spot was more diffuse than in the absence of ammonia, but its R_F was distinct from that of other dibasic amino acids (3). The lysine spot was not separated from ornithine.

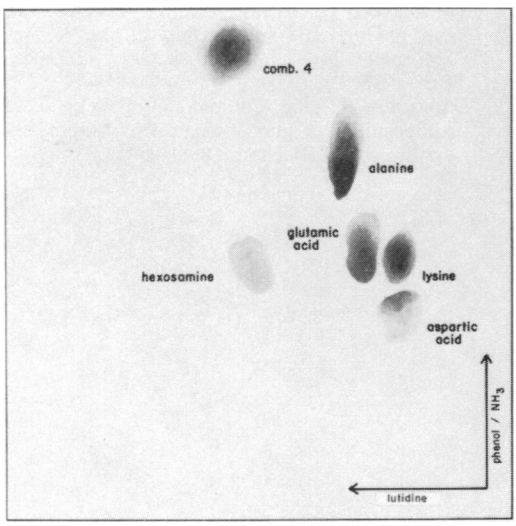

FIG. 1. *Diagram of a typical chromatogram showing the amino acid and amino sugar pattern in Actinomyces bovis P2S.*

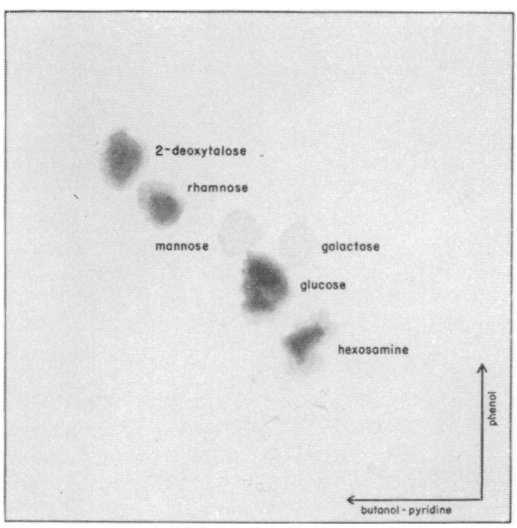

FIG. 2. *Diagram of a typical chromatogram showing the sugar and amino sugar pattern in Actinomyces bovis P2S.*

The procedure originally described by Cummins and Harris (6) provided a more definitive resolution and identification of amino acids present in the cell wall; minor contaminating amino acids may be present in the short procedure described here. Attempts to obtain a more complete removal of contaminating proteins or sugars from whole cells were made by use of glacial acetic acid and liquid phenol. The experimental results showed the one-step NaOH extraction procedure to be the most effective overall procedure, although for sugars the use of liquid phenol was preferred. In addition, as described by Cummins and Harris (6), the short period of hydrolysis with HCl does not release the maximal amount of lysine. Resolution of the lysine spot into ornithine and lysine, which is characteristic of *A. israelii*, is not essential for identification of these species, since they are suitably identified by the combination of the sugar and amino acids chromatograms. Repeated examination of the strains tested has shown that the method described here permits a reliable separation of these and closely related species. The method can be used as a routine laboratory procedure requiring a minimal amount of equipment and effort.

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