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Morphological, Biochemical, and Serological Studies on 64 Strains of *Actinomyces israelii*

JOHN M. SLACK, SANDRA LANDFRIED, AND MARY A. GERENCSER
Department of Microbiology, Medical Center, West Virginia University, Morgantown, West Virginia 26506

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A comparative study of 64 strains of *Actinomyces israelii* was done with the use of techniques standardized by the Subgroup on Taxonomy of the Microaerophilic Actinomycetes. Emphasis was placed on the range of variation to assist recognition of clinical isolates and aid in differentiation from *Actinomyces*-like organisms. None of the strains was positive for catalase or indole, or in the Voges-Proskauer test; 90% were methyl red-positive and 62% were nitrate-positive. Acid was produced from: glucose (100%), xylose (100%), salicin (98%), raffinose (95%), lactose (89%), cellobiose (83%), mannose (78%), arabinose (76%), inositol (58%), mannitol (48%), starch (31%), glycogen (0%), glycerol (0%), and rhamnose (0%). *A. israelii* can be identified by the fluorescent-antibody method, but there is no single morphological or biochemical characteristic which can be used for its identification. By both fluorescent-antibody and gel-diffusion techniques, the serological classification of *A. israelii* group D with serotypes 1 and 2 was verified. Eleven serotype 2 strains were compared morphologically, biochemically, and serologically with 53 serotype 1 strains. All but two of the serotype 2 strains produced viscous growth in broth and none fermented arabinose.

Identification of *Actinomyces israelii* requires differentiation from other species of *Actinomyces* and from morphologically similar organisms. Difficulties may arise because the organism can be difficult to grow or maintain and because the range of strain variation (morphological, biochemical, and serological) is poorly defined. *A. israelii* was distinguished from *A. bovis* by Erikson (3), Thompson (22), and Pine et al. (14), and the principal characteristics distinguishing it from *A. naeslundii*, *A. odontolyticus*, *A. viscosus* [*Odontomyces viscous* (9) is renamed *Actinomyces viscosus* (Georg et al., *to be published*)], *A. propionicus*, and *A. eriksonii* have been described in a number of publications (1, 4-6, 9). With the exception of the work of Howell et al. (10), only limited numbers of strains have been included in any one study. Further, no comparative study has combined serology, morphology, and physiology of a large number of strains and included isolates from different countries.

Identification of *Actinomyces* serologically was difficult, despite application of all the common techniques, until the introduction of fluorescent-antibody (FA) and gel-diffusion procedures. These have successfully achieved specificity and have overcome the inherent difficulties in applying serological methods to filamentous organisms.

The FA technique was first applied to *Actinomyces* by Slack and Moore (Bacteriol. Proc., p. 142, 1960), with the eventual establishment and revision of four serological groups by Slack and Gerencser (18). *A. israelii* was designated as serological group D. The species specificity of the FA technique was confirmed by Lambert et al. (13), who also reported two strains of *A. israelii* belonging to a second serotype. Blank and Georg (2), using FA, demonstrated the *A. israelii* serotypes 1 and 2, as well as other *Actinomyces*, in material from human tonsils.

The gel-diffusion technique, first used in studying *Actinomyces* by King and Meyer (11), has been used to identify *A. israelii* (13) and to indicate generic relationships (12, 19); however, the technique is ordinarily less species specific than the FA test (13). Landfried (M.S. Thesis, West Virginia Univ., Morgantown, 1966) was able to place *Actinomyces* cultures into serological groups corresponding to those determined by FA, but observed more cross-reactions between groups with gel-diffusion than were found with the FA test. Pirtle et al. (15) reported isolation of a water-soluble, heat-stable antigen from culture supernatant fluid of *A. israelii*, which reacted specifically with *A. israelii* antiserum in gel-diffusion tests.

Holm (8), working with human isolates of *Actinomyces*, described a group 1 which was typical *A. israelii* and a group 2 which produced viscous growth in broth, required CO_2, and did not ferment arabinose. He considered this latter group a different species, but he did not give it a name. Howell et al. (10) differentiated three groups of *A. israelii*, primarily on the basis of fermentation reactions, but they indicated that this was not a reliable basis for differentiation, and these groups did not correspond to those of Holm. Thus, there has been no reliable way of grouping or typing the *Actinomyces* until the introduction of reproducible serological procedures.

This paper presents the results of detailed morphological, biochemical, and serological studies on 64 strains of *A. israelii*. Whenever possible, standardized techniques recommended by the International Subgroup on the Taxonomy of Microaerophilic Actinomycetes (17) were used. The combined approach more clearly defines *A. israelii* but, perhaps of greater importance, indicates the range of variation within these different reactions, which might be observed with any given culture. Serotype 2 of *A. israelii* (group D), which had been tentatively reported (13), is established by characterizing 11 strains and comparing their reactions with those of serotype 1 strains.

MATERIALS AND METHODS

Organisms. Sixty-four strains of *A. israelii* from the United States and several foreign countries were studied. The West Virginia University (WVU) collection numbers and sources of these strains are given in Table 1.

Identity of the cultures. When received, the majority of the cultures were designated as *A. israelii*, but some were labeled only as *Actinomyces* and others as *A. bovis*, *A. naeslundii*, or *Actinobacterium cellulitis*. All cultures from the Institut Pasteur were named *Actinobacterium*. Our initial criterion for identity was a 2+ or better FA reaction with one or both WVU 30 (ATCC 10048) and WVU 46 (ATCC 12102) specific conjugated antiserum. Determinations of other characteristics followed.

Stained smears and dark-field preparations. Smears were made from 3- and 7-day-old cultures grown in Fluid Thioglycollate Broth (Difco) and stained by the Gram method (20). Dark-field microscopy was done on suspensions from the same cultures, with the use of a Leitz D 1.20 condenser and × 95 fluorite oil objective. Photographs were taken with a Leitz Orthomat Automatic camera.

Microcolonies. Brain Heart Infusion (BHI) plates were streaked, placed in an atmosphere of 95% N_2-5% CO_2, and examined microscopically after 18 to 24 hr and 48 hr of incubation at 35 C. Small blocks of agar were transferred onto slides to assist microscope observation at magnifications of 125 to 950 times. Photographs were taken with a Leitz Orthomat Automatic camera.

Macrocolonies. BHI plates were streaked and incubated as above, and colonies were examined and photographed by use of an AO dissecting microscope at a magnification of 7 to 50 times after 7 and 14 days of incubation at 35 C.

Growth in broth. Fluid Thioglycollate Medium (Difco) and Actinomyces Broth (BBL) were inoculated, incubated for 7 days at 35 C, and observed for the type of growth.

Determination of oxygen requirements. Suspensions prepared from 3-day-old Actinomyces Broth (BBL) cultures were used to inoculate BHI slants which were then incubated aerobically, aerobically with CO_2, anaerobically, and anaerobically with CO_2. The details of this technique were described by Gerencser and Slack (6).

Biochemical tests. The biochemical tests included catalase, nitrate reduction, indole production, methyl red (MR), Voges-Proskauer (V-P), hydrogen sulfide production, starch hydrolysis, and gelatin hydrolysis. The production of acid from carbohydrates was tested with Actinomyces Fermentation Broth (BBL) as the basal medium. Arabinose, xylose, glucose, mannose, cellobiose, lactose, raffinose, glycogen, glycerol, mannitol, salicin, starch, rhamnose, inositol, and sorbitol were tested. The techniques used in the biochemical tests are described in a report of the International Subgroup on the Taxonomy of Microaerophilic Actinomycetes (17).

Antisera. Methods of antigen preparation and immunization schedules were the same as described by Gerencser and Slack (6) except that the antigen was mixed (1:1) with Freunds Incomplete Adjuvant (Difco) for the intradermal injections. Globulin fractions of the antisera were obtained by ammonium sulfate precipitation and conjugated with fluorescein isothiocyanate (FITC). The excess fluorescein was removed by filtration through a Sephadex G-25 column. Conjugated antiserum was prepared for *A. israelii* WVU 30 (ATCC 10048), WVU 46 (ATCC 12102), WVU 307, WVU 339, and WVU 390.

Working titers of conjugates. Conjugated antisera were titered by staining the homologous strain with serial twofold dilutions made in FA Buffer (Difco). The titer of the antiserum was the highest dilution giving 4+ fluorescence. The "working titer" for each antiserum was one-half this titer (21); that is, a serum with 4+ staining at 1:160 had a working titer of 1:80.

Staining of smears for FA. The cultures were grown in Actinomyces Broth (BBL) for 7 days, centrifuged, and resuspended in 0.5% Formalin-treated saline. Smears were made from these suspensions, allowed to air-dry, and gently heat-fixed. Conjugates were applied to the dried smears, and the slides were incubated in a moist chamber at room temperature for 30 min. They were then washed in phosphate-buffered saline for 10 min, counterstained with Evans Blue dye for 10 min, and finally rinsed for 1 min each in two changes of *p*H 9.0 buffer (23). The smears were mounted in buffered glycerol-saline and ex-

TABLE 1. *Laboratory numbers and sources of cultures of Actinomyces israelii*

W V U no.	Source	Original no. or designation
30	ATCC 10048	Emmons, 1829
33	Sylvia King, Chicago, Ill.	CC 3
46	ATCC 12102	Howell, 277
47	ATCC 12103	Howell, 287
69	Goldsworthy, Australia	Hunter
70	Goldsworthy, Australia	Butler
72[a]	Goldsworthy, Australia	Lemke
159	Howell, National Institutes of Health	295
184[a]	Howell, National Institutes of Health	C 479
213	Negroni, Argentina	146
219	Prevot, Institut Pasteur, Paris, France	1031
231	Prevot	1217
232	Prevot	1597 A
233	Prevot	1641 A
236	Prevot	2259
237[a]	Prevot	2018
238	Prevot	1960
243	Prevot	932 A
287	Prevot	1222
288	Prevot	1200 A
290	Prevot	1025
299	Holm, Serum Institut, Denmark	1061/40–41
300	Holm	297/45–46
301[a]	Holm	268/45–46
302	Holm	2129/40–41
303	Holm	39/46–47
304[a]	Holm	161/45–46
305	Holm	186/45–46
306	Holm	104/45–46
307[a]	Holm	8/46–47
308	Holm	1919/40–41
310	Holm	2481/40–41
311	Holm	1435/40–41
312[a]	Holm	1406/40–41
314	Holm	170/45–46
315	Holm	234/45–46
316	Holm	195/45–46
317	Holm	169/45–46
318	Holm	54/46–47
319	Holm	1341/40–41
320	Holm	175/45–46
321	Holm	1654/40–41
322	Holm	818/40–41
324[a]	Holm	311/45–46
325	Holm	260/45–46
326[a]	Holm	1452/40–41
327	Holm	223/45–46
333	Weed, Mayo Clinic	1016
339	Porteus, Scotland	Wills
345	Lentze, Germany	I (Harsotte)
370	Negroni, Argentina	257
384	Duda, Oakland Hospital, Pa.	Berkley
389	Meyer, Univ. of Illinois	AB
390	Meyer	Bradley
396	Slack, West Virginia Univ.	Ratliff
448[a]	Georg, National Communicable Disease Center	X695

TABLE 1.—*Continued*

W V U no.	Source	Original no. or designation
450[a]	Slack	Moore
452	ATCC 10049	Emmons, 1833
457	ATCC 12103	Howell, 287
458	ATCC 12836	CDCB-99 (bovine)
460	ATCC 12597	CDC MY-565-55
461	ATCC 13031	Meyer (bovine)
525	Slack	4B-37
575	Slack	7-45

[a] Serotype 2 strains; all others were serotype 1.

amined under a Leitz Ortholux microscope equipped with an HBO 200 mercury vapor lamp, cardioid dark-field condenser, BG 12 primary filter, and an OG-1 eye-piece filter. Fluorescence intensity was graded on a 0 to 4+ scale, but only reactions graded as 2+ or better were considered positive.

Gel-diffusion technique. Antiserum was prepared against whole cells as described above. The antigen preparation was made by adding 2 volumes of acetone to 1 volume of culture supernatant fluid, refrigerating the mixture overnight, and dissolving the recovered precipitate in distilled water. The gel-diffusion medium was prepared with FTA-Hemagglutination Buffer (BBL) containing 0.7% Special Agar-Noble (Difco) and 1:10,000 thimerosal. Micro two-dimensional gel-diffusion tests were set up by pipetting 8 ml of the melted medium onto a slide (5 × 7.6 cm), allowing the agar to harden, and then punching the wells. The slide was placed in a petri dish and sufficient FTA buffer was added to contact the edges of the agar. After overnight refrigeration, the agar was removed from the wells by suction. Antiserum was added to the 8-mm central well, and soluble antigens were placed in the 2.5-mm peripheral wells, the latter being 5 mm from the central well. Incubation was at room temperature, and readings were made at 2, 3, and 4 days.

RESULTS

Cellular morphology. The organisms showed predominately V, Y, and T forms after growth for 72 hr in Thioglycollate Broth (Fig. 1). They were gram-positive and sometimes stained uniformly but more often stained irregularly, giving a beaded appearance (Fig. 2). Most cultures exhibited elongated branching filaments which also stained irregularly (Fig. 3). The filaments occasionally were wavy, had terminal bulbous ends, or occurred as intertwined masses (Fig. 4).

Microcolony morphology. Two-thirds of the cultures produced a filamentous microcolony made up of angular branching filaments (Fig. 5) or of elongated multiply branching filaments (Fig. 6). The colonies which have filaments radiating from a single point with little or no "center" are representative of the "spider colonies" usually described as characteristic of *A. israelii*. "Spider" colonies could not always be demonstrated in these cultures. About one-third of the 64 strains produced small compact colonies (Fig. 7) which were not suggestive of their identity as *A. israelii*.

Macrocolony morphology. Mature colonies varied from smooth to irregular, and after 14 days of incubation ranged from 0.5 to 2.0 mm in size. Occasionally, colonies 3 mm in diameter were observed. The smooth colonies (Fig. 8 and 9) were circular, slightly irregular, convex, pulvinate or umbonate, entire, opaque, white to gray-white, and soft. Such colonies were formed by one-third of the cultures and could result from either small compact or filamentous microcolonies. Rough colonies (Fig. 10) were circular or irregular, convex, pulvinate or heaped, finely granular to granular, erose to lobate, opaque, friable, and usually adherent. The surface of these colonies was often highly convoluted, giving a "pebbly" or "bread-crumb" appearance. Central depressions were often deep enough to appear as holes, producing "raspberry-like" colonies (Fig. 11). Rough colonies were produced by 48 of the strains, and there was no correlation between microcolony type and the shape or size of the mature colony. Usually, a given culture would produce either smooth or rough colonies, although some cultures produced both on the same plate.

Growth in broth. In Actinomyces Broth or BHI broth, most strains produced either a fine or coarsely granular growth which usually sedimented, leaving a clear supernatant fluid, but in Thioglycollate Broth the growth occurred as discrete granules throughout the medium. Nine strains gave a distinctly different type of growth which was both diffuse and viscous. The degree of viscosity varied, but the growth was always visibly different from the granular cultures (Fig. 12).

Oxygen requirements. The oxygen requirements of 63 strains were determined (Table 2).

All strains produced maximal (3+) growth when they were incubated anaerobically with or without added CO_2. Under these test condltions, 50 strains also showed good (2+ to 3+) growth aerobically with CO_2, and 12 strains showed good growth when incubated aerobically without CO_2.

Biochemical reactions. The results of the biochemical tests are shown in Table 3 for the entire

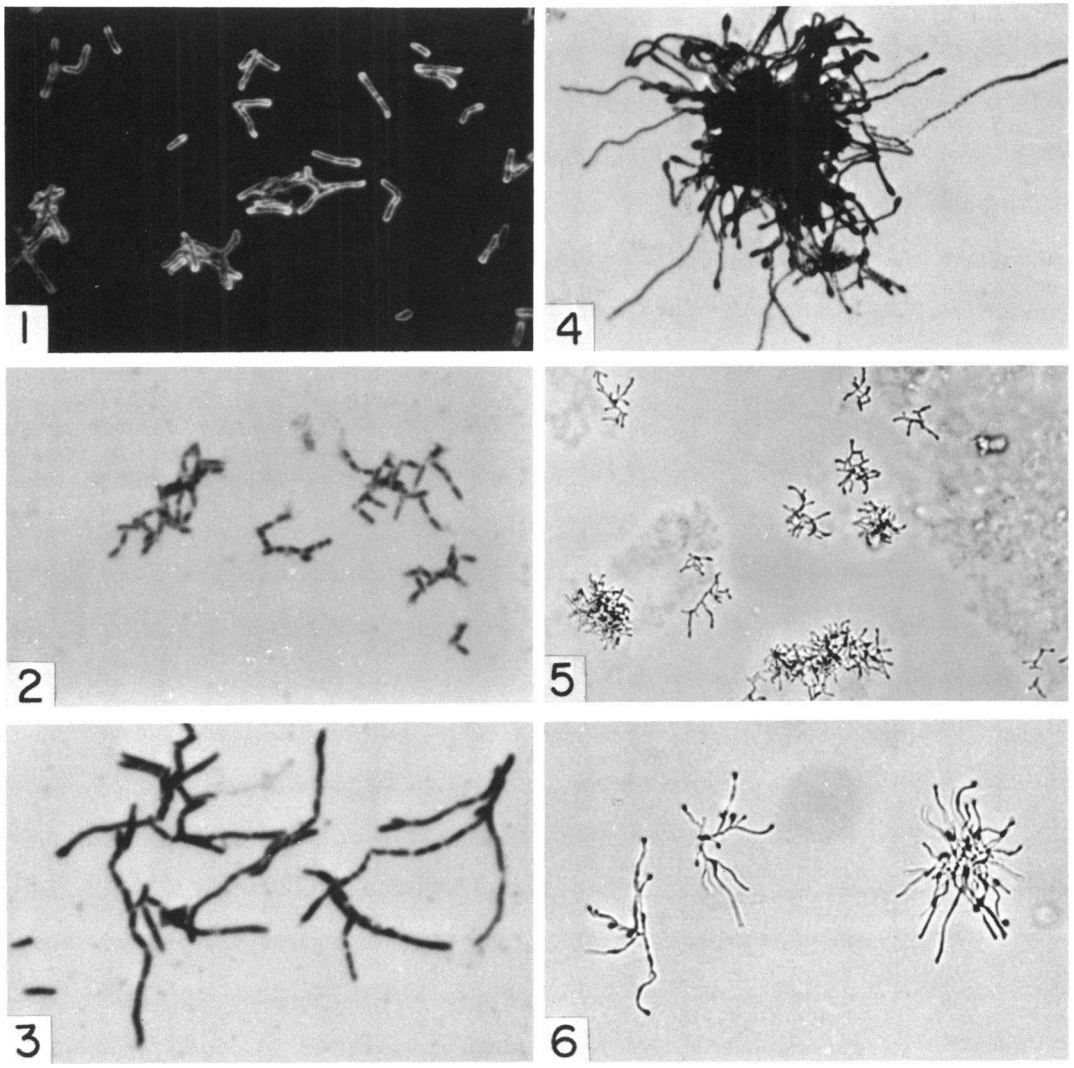

FIG. 1. *Dark field from 72-hr growth in Thioglycollate Broth, showing V and Y forms and an indication of granular cytoplasm.* × *1,260.*

FIG. 2. *Gram stain from 72-hr growth in Thioglycollate Broth, showing irregularly stained rods of variable length and angular arrangements.* × *1,580.*

FIG. 3. *Gram stain from 72-hr growth in Thioglycollate Broth, showing elongated filaments, branching, and some irregular staining.* × *1,890.*

FIG. 4. *Gram stain from 72-hr growth in Thioglycollate Broth, showing a mass of branching intertwined filaments. Some filaments irregularly stained and some with small bulbous ends.* × *1,240.*

FIG. 5. *Unstained 24-hr microcolonies of A. israelii growing on BHI, showing filaments with multiple short angular branches. Resembles A. naeslundii microcolonies.* × *410.*

FIG. 6. *Unstained 24-hr microcolonies growing on BHI, showing branching, filamentous or "spider" colonies with no distinct center. Similar colonies may be formed by A. propionicus, rough A. odontolyticus, and rough A. bovis.* × *410.*

group of 64 strains and for the strains arranged according to the serotypes which will be described later in this report.

All 64 strains were catalase-negative. Since catalase may be inducible only under aerobic conditions, the growth on the BHI slants used for the oxygen requirement tests was tested for catalase production.

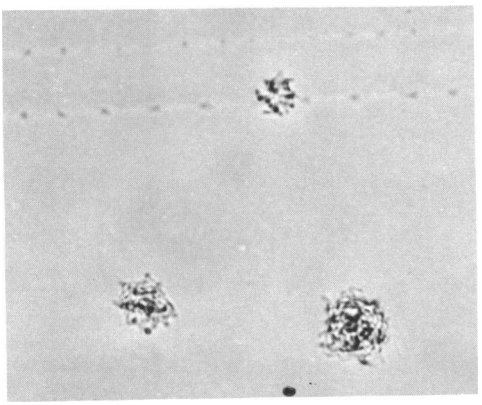

FIG. 7. *Small, compact, slightly elevated 24-hr microcolonies on BHI. Not indicative of A. israelii.* × *500.*

None of the cultures produced indole or acetoin. Approximately 90% were M R-positive when tested after 14 days of incubation. If the M R test was done after 7 days of incubation, 20 of the 56 positive strains gave doubtful (orange in color) results. Thirty-nine strains reduced nitrate in 7 days. All of the cultures grew well in the Indole-Nitrite Medium (BBL) used for the tests, so the negative results cannot be attributed to poor growth. It should be noted that 31 of the negative cultures were from foreign countries and only 3 were from the U.S. If the strains are grouped by serotype without regard to source, only 27% of the type 2 strains reduced nitrate in 7 days as compared with 68% of the type 1 strains.

Hydrolysis of potato starch was variable, and two types of reactions were produced after 14 days of incubation. Small, colorless zones were observed around the growth of 26 strains. These zones ranged up to 8 mm in width from the edge of the growth to the edge of the zone. The actual

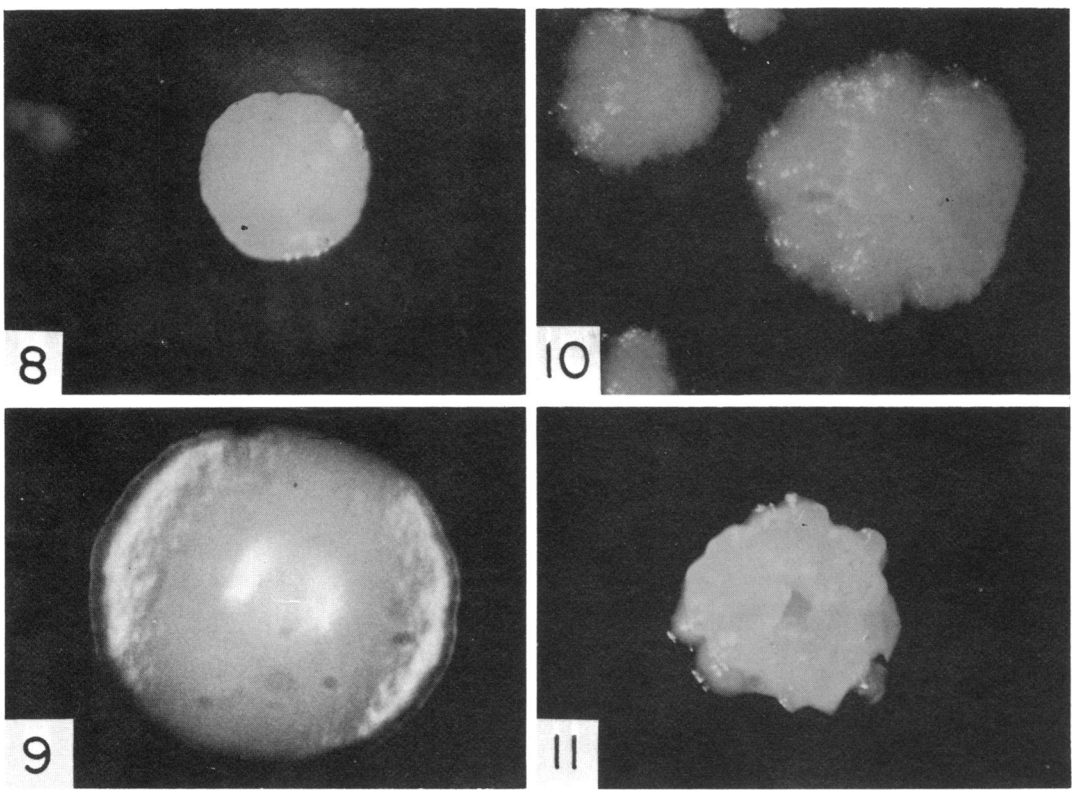

FIG. 8–11. *Colonies on BHI, 14 days. (8) Smooth, pulvinate, entire edge;* × *16. (9) Smooth, umbonate, with narrow translucent edge;* × *40. (10) Rough, umbonate with deep radial striations at periphery; erose edge;* × *32. (11) Rough, heaped, with central crater "raspberry-like";* × *20.*

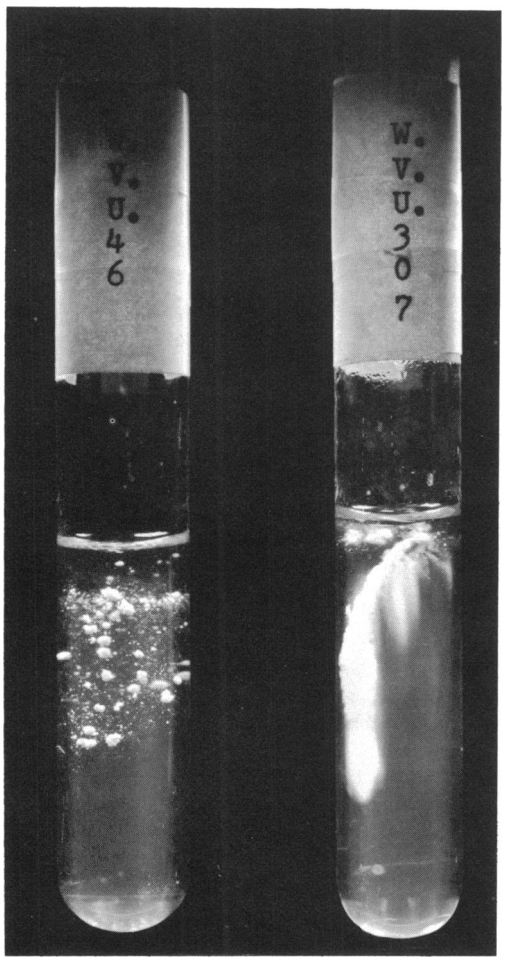

FIG. 12. *Seven-day growth in Thioglycollate Broth. A. israelii 46 shows the typical granular growth and 307 demonstrates the viscous growth observed in the majority of serotype 2 cultures.*

width of the zone was highly dependent on the amount of growth along the streak. Thirty-two additional strains produced zones of similar size, which were transparent but distinctly reddish-pink in color. These strains were recorded as negative even though they produced some degree of hydrolysis.

No strains hydrolyzed gelatin in 30 days when tested in a tube of nutrient gelatin. Growth in this medium was generally very poor. When the gelatin-agar plate method was used, 32 strains produced zones of hydrolysis up to 5 mm in width (edge of growth to edge of zone) in 14 days. These results were dependent upon obtaining heavy growth along the streak.

All 64 *A. israelii* strains grew well in tubes of Actinomyces Fermentation Broth (BBL) closed with carbonate-pyrogallol seals. The fermentation patterns were more reproducible when the tests were held for 14 days; the fermentation reaction of arabinose in particular was often ambiguous at 7 days but acidic at 14 days. All strains produced acid without gas from glucose and xylose, 98% fermented salicin, and 97% fermented raffinose. No strain fermented glycogen, glycerol, or rhamnose, and only 11% fermented sorbitol. There was more variation in the other carbohydrates tested, but the majority of strains fermented lactose (89%), cellobiose (84%), mannose (78%), arabinose (64%), and inositol (63%). Approximately 50% of the cultures fermented mannitol and 33% fermented starch. The fermentation reactions were very similar when separated according to serotype, except that none of the type 2 strains fermented arabinose.

Reactions of A. israelii and FITC-conjugated antiserum to other species and genera. None of the *A. israelii* strains stained with conjugated antiserum for *A. bovis* (ATCC 13683), *A. eriksonii* (ATCC 15423), *A. odontolyticus* (ATCC 17929), *A. parabifidus* (ATCC 17930), *A. propionicus* (ATCC 14157), *Rothia dentocariosus* (ATCC 17931), *Bacterionema matruchotti* (ATCC 14266),

TABLE 2. *Results of oxygen-requirement determinations*

Test conditions	Reaction[a]	No. of cultures		
		Total	Serotype 1	Serotype 2
Aerobic	0	20/63[b]	9/52	11/11
	1+	31/63	31/52	0/11
	2+	10/63	10/52	0/11
	3+	2/63	2/52	0/11
Aerobic + CO_2	0	5/63	0/52	5/11
	1+	8/63	6/52	2/11
	2+	8/63	7/52	1/11
	3+	42/63	39/52	3/11
Anaerobic	0	0/63	0/52	0/11
	1+	0/63	0/52	0/11
	2+	1/63	1/52	0/11
	3+	62/63	51/52	11/11
Anaerobic + CO_2	0	0/63	0/52	0/11
	1+	0/63	0/52	0/11
	2+	0/63	0/52	0/11
	3+	63/63	52/52	11/11

[a] Reactions: 3+, the best growth under one of the culture conditions; 2+, medium growth in comparison to the best; 1+, slight growth in comparison to the best; 0, no growth.

[b] No. of strains giving indicated reaction/no. of strains tested.

TABLE 3. *Biochemical reactions and carbohydrate fermentation of A. israelii*

Test	No. of cultures		
	Total	Serotype 1	Serotype 2
Biochemical tests[a]			
Catalase	0/64	0/53	0/11
Indole	0/63	0/52	0/11
Nitrate	39/63	36/52	3/11
Methyl red	57/63	46/52	11/11
Voges-Proskauer	0/63	0/52	0/11
Starch hydrolysis[b]	26/63	25/52	1/11
Gelatin hydrolysis			
Tube method	0/63	0/52	0/11
Plate method[c]	32/63	32/52	0/11
Carbohydrate fermentations[d]			
Arabinose	41/64	41/53	0/11
Xylose	64/64	53/53	11/11
Glucose	64/64	53/53	11/11
Mannose	50/64	39/53	11/11
Cellobiose	54/64	43/53	11/11
Lactose	57/64	46/53	11/11
Raffinose	62/64	53/53	9/11
Glycogen	0/64	0/53	0/11
Glycerol	0/64	0/53	0/11
Inositol	39/62	31/51	8/11
Mannitol	31/64	24/53	7/11
Salicin	63/64	52/53	11/11
Sorbitol	7/61	7/50	0/11
Rhamnose	0/61	0/50	0/11
Starch	21/64	19/53	2/11

[a] No. of strains positive/no. of strains tested. Indole, nitrate, and Voges-Proskauer, final reading at 7 days. Methyl red, starch, and gelatin, final reading at 14 days.

[b] Small zones of hydrolysis up to 8 mm.

[c] Small zones of hydrolysis up to 5 mm.

[d] No. of strains producing acid in 14 days/no. of strains tested.

Corynebacterium pyogenes (ATCC 19411), or *C. hemolyticum* (ATCC 9345).

None of the strains was stained by the working titer (1:80) of *A. naeslundii* (ATCC 12104) antiserum. However, 36 strains were positive with the serum undiluted and 11 strains were positive with a 1:10 dilution. Also, all strains were negative at the working titer (1:80) of *A. viscosus* antiserum, but 10 were FA-positive with undiluted antiserum and 4 were positive at a 1:40 dilution. However, none of these reactions was greater than 2+ in intensity.

Reactions of A. israelii FITC-conjugated antiserum with other species and genera. With the exception of *A. naeslundii* and *A. viscosus*, none of the other species of *Actinomyces* or species of the other genera reacted with either of the *A. israelii* antisera (Table 4). Certain *A. naeslundii* strains fluoresced at a higher dilution with *A. israelii* strain 46 than with *A. israelii* strain 30 antiserum, indicating an antigenic difference between the two strains.

Serotype 2 of A. israelii. Initially, conjugated antiserum to strain 30 was used to screen unknown cultures for identification as *A. israelii*. However, in investigating cultures obtained from Per Holm of the Statens Seruminstitut, Copenhagen, Denmark, six cultures were found which did not fluoresce with strain 30 antiserum at its working titer. Further investigations showed that three of these cultures stained at lower dilutions of strain 30 antiserum and that all of them stained with strain 46 conjugated antiserum, five strains at its working titer of 1:80 and one at 1:40. This suggested that these six strains represented a second serotype and that there were minor antigenic differences between *A. israelii* 30 and 46.

Conjugated antiserum was prepared against serotype 2 by use of *A. israelii* strain WVU 307. The results for all of the strains with this and four serotype 1 antisera are shown in Table 5. A total of 11 strains were established as serotype 2. All fluoresced with 307 antiserum and varying numbers reacted with the type 1 antisera. All 53 of the type 1 strains were positive with the four type 1 antisera, and 14 of these fluoresced with the type 2 antiserum at its working titer, although in many cells the fluorescence appeared as bright (4+) spots along the walls of the cells and not as the solid bright staining of the type 2 cells.

TABLE 4. *Reactions of A. israelii antiserum with other species or genera*

Antigen	FITC-conjugated antisera						
	A. israelii WVU 30			*A. israelii* WVU 46			
	Undiluted	1:10	1:20	Undiluted	1:10	1:20	1:40
Actinomyces bovis	0/7[a]	—	—	0/7	—	—	—
A. eriksonii	0/3	—	—	0/3	—	—	—
A. naeslundii	7/11	1/11	—	6/8	6/8	6/8	—
A. odontolyticus	0/3	—	—	0/3	—	—	—
A. parabifidus	0/1	—	—	0/1	—	—	—
A. propionicus	0/7	—	—	0/7	—	—	—
A. viscosus	4/6	0/6	—	0/6	—	—	—
Rothia dentocariosus	0/4	—	—	0/4	—	—	—
Bacterionema matruchotti	0/4	—	—	0/4	—	—	—
Corynebacterium pyogenes	0/3	—	—	0/3	—	—	—
C. hemolyticum	0/1	—	—	0/1	—	—	—

[a] No. of strains positive/no. of strains tested.

TABLE 5. *Serological reactions of 64 strains of A. israelii with serotypes 1 and 2 antisera*

Culture	FITC-conjugated antisera[a]				
	Type 1, 30	Type 1, 46	Type 1, 390	Type 1, 339	Type 2, 307
Type 1...	53/53[b]	53/53	53/53	53/53	14/53
Type 2...	1/11	8/11	10/11	0/11	11/11

[a] Antisera used at working titer.
[b] No. of strains positive/no. of strains tested.

TABLE 6. *Serological reaction of 64 strains of A. israelii with sorbed serotypes 1 and 2 antisera*

Culture	Conjugated antisera (1:10)	
	Type 1 (46) sorbed with type 2 (307) cells	Type 2 (307) sorbed with type 1 (46) cells
Type 1.....	53/53[a]	0/53
Type 2.....	9/11	11/11

[a] No. of strains positive/no. of strains tested.

TABLE 7. *Sorption tests between three A. israelii serotype 1 strains*[a]

Antiserum	Sorbed with cells of strain	Antigen		
		30	46	390
30	—	4+	4+	4+
	46	Neg	Neg	NT
	390	Neg	NT	Neg
46	—	4+	4+	4+
	30	Neg	3–4+	3–4+
	390	NT	Neg	Neg
390	—	4+	4+	4+
	30	Neg	3–4+	3–4+
	46	NT	Neg	Neg

[a] NT = not tested; neg = negative.

Reciprocal sorptions were done with conjugated antisera of type 1 (30 and 46) and type 2 (307) and the respective antigens. The homologous antigens completely sorbed their own antisera. Type 1 antiserum sorbed with type 2 cells stained type 1 but not type 2 cells. Type 2 antiserum sorbed with type 1 cells (30 or 46) stained type 2 but not type 1 cells. These results clearly established the two serotypes. The culture (WVU 450, CDC X695) described by Lambert et al. (13) as a serotype 2 was used to sorb antiserum 307. The resulting serum stained neither 307 or 450 cells, which establishes that the second serotype reported here and that reported by Lambert et al. (13) are the same.

The above sorbed antisera were tested with all 64 strains (Table 6). Type 1 cultures reacted only with type 1 sorbed antiserum and type 2 strains stained only with the sorbed type 2 antiserum.

Serotype 1 of A. israelii. The above results show antigenic homogeneity among the type 1 strains; however, as type 2 cells (Table 5) showed a variation in reaction to these antisera, there is an indication of antigenic differences. Thus, sorption studies were done with type 1 strains (Table 7). Strains 46 and 390 appeared to be identical but strain 30 lacked at least one antigen present in the other two strains. When all 53 type 1 strains were tested with the strain 46 antiserum sorbed with strain 30 cells, only 3 strains (30, 159, and 339) failed to stain, indicating that 95% of the type 1 strains are antigenically similar to strain 46. Although these antigenic differences are demonstrable among the type 1 strains, they are considered minor and not great enough to separate these strains into serotypes.

Gel-diffusion test. When the technique described earlier was used and tests were incubated for 4 days at room temperature, the homologous reactions of *A. israelii* formed three or four strong sharp lines which were sufficiently characteristic to identify the species. These results parallel those obtained with FA (if incubation was prolonged a total of six or seven lines would form).

The homologous reactions also confirmed the presence of the two serotypes of *A. israelii*, as well as the previously indicated minor antigenic differences within the serotype 1 strains. Type 1 strains, as represented by reactivity with 46 antiserum, formed two or three lines of identity with other type 1 strains. The three strains (30, 159, and 339), which were antigenically different from most other type 1 strains by FA, possessed only one of these antigens, as determined from the formation of only one line of identity with strain 46 antiserum. In cross-reactions between type 1 strains and type 2 antiserum or type 2 strains and type 1 antiserum, the reactions were weak, but usually one or two faint lines could be seen. In the reactions with their own type antisera, the lines were strong and usually well defined. With antigen preparations from the other species of *Actinomyces*, *Corynebacterium pyogenes*, and *Corynebacterium acnes*, one or two lines could often be demonstrated.

DISCUSSION

The speciation of *Actinomyces* cultures is often considered very difficult. For example, on the basis of published descriptions, Rasmussen et al. (16) could identify only 11 of 37 strains of

catalase-negative "diphtheroids" isolated from the mouth. A number of recent papers (4–6, 9) have discussed the differential characteristics of *Actinomyces* species but have usually included only a few strains for comparative purposes.

The primary purpose of this study was to provide help in identifying *A. israelii* by better defining the range of variation within the species. The comparative references to other species included in this discussion are based on our studies of a few strains of each species by the same techniques described above for *A. israelii* and on results in the literature. In identifying *Actinomyces* from human sources, the species most likely to be confused with *A. israelii* are *A. propionicus* and *A. naeslundii*. *A. propionicus* produces infection in man, but the pathogenicity of *A. naeslundii* is still in question. *A. bovis* has not been found in human clinical material to our knowledge, but *A. israelii* has been found in animals (14) so that in material from animal lesions *A. israelii* must be distinguished from *A. bovis*.

In identifying a bacterial culture, microscopic morphology and Gram reaction are usually the first criteria used. A Gram-stained smear showing gram-positive, filamentous, branching rods immediately suggests the possibility of *Actinomyces*, but *Actinomyces* cultures frequently exhibit only "diphtheroidal" morphology similar to that of *Corynebacterium*, *Propionibacterium*, *Ramibacterium*, and *Rothia*. Therefore, *Actinomyces* should always be considered in identifying such cultures.

The first characteristic that is useful for species differentiation of *Actinomyces* is microcolony morphology. The filamentous microcolony with no distinct central area (so-called "spider" colony) is considered typical of *A. israelii* (14). However, *A. propionicus*, rough *A. odontolyticus*, and rough *A. bovis* may produce indistinguishable microcolonies. Some strains of *A. israelii* produce microcolonies composed of shorter, angularly branched filaments very similar to the microcolonies produced by some strains of *A. naeslundii*. None of the *A. israelii* microcolonies had the dense center with radiating filaments which is considered typical of *A. naeslundii*. Of the 64 cultures, 20 produced small, compact colonies without a filamentous edge, which were not indicative of an *A. israelii* culture. Therefore, microcolony type has limitations as an identifying characteristic of *A. israelii*.

Mature colonies of *A. israelii* are most frequently irregular, rough in appearance, and "heaped-up," but *A. israelii* may produce smooth, entire, convex colonies. It is important to have an awareness of this range of colonial morphology when selecting colonies from isolation plates and to realize that similar mature colony types are encountered with *A. naeslundii* and *A. propionicus*.

A. israelii is facultative, with all strains growing best under anaerobic conditions. The majority of these strains grew aerobically, but there was no attempt to determine their ability to grow aerobically through repeated transfers or their ability to initiate growth from a small inoculum. Many strains were stimulated by CO_2, particularly when grown aerobically, although 10% grew just as well without CO_2. These results are in general agreement with those reported by Howell et al. (10). In comparison, *A. eriksonii* is anaerobic, *A. viscosus* is aerobic, and both *A. naeslundii* and *A. propionicus* are more oxygen-tolerant than is *A. israelii*.

On the basis of the reactions of these 64 strains, the biochemical characteristics of *A. israelii* may be summarized as follows: catalase-negative (100%), indole-negative (100%), V-P-negative (100%), M R-positive (90%), nitrate positive (62%), starch hydrolysis variable, and gelatin hydrolysis variable; acid is produced from glucose (100%), xylose (100%), salicin (98%), raffinose (95%), lactose (89%), cellobiose (83%), mannose (78%), arabinose (76%), inositol (58%), mannitol (48%), and starch (31%). These results require comment as to the usefulness of the various tests in identification.

The catalase test is frequently used for distinguishing *Actinomyces* from anaerobic *Corynebacterium* species which are catalase-positive. Other genera, *Rothia* and *Propionibacterium*, which may resemble *Actinomyces* are also catalase-positive, as is one species of *Actinomyces*, *A. viscosus*. Anaerobically grown cultures of *Rothia* and *A. viscosus* may be catalase-negative. Therefore, aerobically grown cultures should be used for this test on all cultures which will grow aerobically. If anaerobically grown cultures are used, it has been suggested (L. Georg, *personal communication*) that they be exposed to air for at least 30 min before doing the catalase test. This procedure has been satisfactory in our hands.

Results of the M R test are highly dependent on incubation time. To obtain positive results with 90% of the strains, 14 days of incubation was necessary. If the tests were done at 7 days, only about one-half of *A. israelii* strains should be expected to give distinctly positive results, and many strains will be doubtful (orange in color).

The nitrate test is useful in distinguishing *A. israelii* from *A. bovis*, which is usually nitrate-negative. It must be remembered, however, that not all strains of *A. israelii* reduce nitrate so that the test cannot be used to exclude *A. israelii* definitely in identifying an unknown culture.

Pine et al. (14) considered the ability of *A. bovis*

to hydrolyze starch rapidly and completely its most striking physiological difference from *A. israelii*. This test is very helpful in separating these species, but the differences are more quantitative than qualitative. For example, 23 of the strains used in this study hydrolyzed starch to some degree, requiring 7 to 14 days to produce small zones of hydrolysis. In contrast, *A. bovis* produces wide zones of hydrolysis which may cover an entire petri plate in 2 to 3 days.

Although *A. israelii* is primarily saccharolytic rather than proteolytic, hydrolysis of gelatin by some strains can be demonstrated if methods more sensitive than the conventional nutrient gelatin tube test are used. *Actinomyces* is described as not liquefying gelatin, so that ability of an unknown culture to do so would be considered sufficient reason for excluding it from the genus. For this reason, it is important to realize that when the gelatin-agar plate technique (7) is used for determining gelatin hydrolysis some strains of *A. israelii* give positive results. *A. propionicus* also hydrolyzes gelatin by this method, but *A. naeslundii* does not.

The fermentation reactions were quite variable, and the 64 strains could have been divided into three subgroups on this basis, as reported by Howell et al. (10). We agree with Howell that this type of subdivision serves no useful purpose, but the fermentation reactions were useful in identification. While the total fermentation pattern (Table 3) must be considered, certain sugars (xylose, raffinose, and cellobiose) are especially helpful. Most *A. israelii* strains ferment all three, and all strains ferment two of the three. Neither *A. naeslundii* nor *A. propionicus* ferments xylose or cellobiose, but they do ferment raffinose. *A. bovis* strains do not ferment raffinose or cellobiose and only occasionally ferment xylose.

The 64 strains fell into two serotypes (53 in serotype 1, and 11 in serotype 2). The FA test was the most reliable method for the determination of types or the identification of the species. Since cross-reactions between the two serotypes are limited, either two sera or a pooled serum should routinely be used for identification. Cross-reactions with other species or genera can be eliminated by appropriate dilution or sorption of the antiserum. Serological identification of either species or types can also be accomplished using culture supernatant fluid and appropriate antiserum in the gel-diffusion test. However, the specificity of this test needs to be improved by devising better methods for antigen and antiserum preparation.

The 11 strains of serotype 2 were quite uniform in their biochemical reactions (Table 3), but they could not be definitely separated from type 1 strains on this basis. However, an *A. israelii*-like culture which produces a viscous growth in broth (two strains were granular) and does not ferment arabinose should be suspected as a serotype 2.

It is obvious from this study that no single morphological or biochemical characteristic is diagnostic for *A. israelii* and that the total pattern of reactions, including serological studies (preferably FA), is necessary for identification. Although in selecting the 64 cultures included in this study serology was the criterion for their identity, this does not mean that serology can always be relied upon in identifying cultures. Additional serotypes of *A. israelii* will probably be found which may have little cross-reaction with the two known types; thus, a strain with the biochemical and morphological characteristics of *A. israelii* could not be excluded from the species on the basis of negative serological reactions. In such cases, the use of cell wall analysis or glucose fermentation end products, or both of these criteria, might be required for definitive identification.

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