JOURNAL OF BACTERIOLOGY, Oct. 1967, p. 875-883

Vol. 94, No. 4
Printed in U.S.A.

Comparative Cell Wall Analyses of Morphological Forms Within the Genus *Actinomyces*

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Received for publication 28 April 1967

Comparative cell wall analyses were made of mycelial and smooth forms of *Actinomyces bovis* and *A. israelii* to determine the changes which occur in the cell wall composition concurrent with a change in morphology, and to evaluate cell wall analyses as a criterion for taxonomic identification within the genus *Actinomyces*. Cell walls of the spider forms of *A. bovis* had little or no aspartic acid and a high hexosamine concentration; cell walls of the smooth forms had a high aspartic acid content and low concentrations of hexosamine. Both forms had large amounts of glutamic acid, alanine, and lysine, as previously reported. A strain of *Actinomyces*, previously identified as *A. naeslundii* on the basis of morphology and aerobic growth characteristics, was found to have the basic cell wall composition of *A. israelii*. When transferred from the *Actinomyces* maintenance broth to a thioglycolate broth, the cells of this strain passed from a mycelial form through a transient filamentous morphology to become diphtheroidal with continued incubation. Concomitantly, the concentrations of glutamic acid relative to alanine decreased, and the hexosamine content increased. Variation in morphology within the species *A. israelii* and *A. bovis* could not be related to any mutual chemical change of their cell walls.

During the past 10 years, considerable progress has been made on the taxonomy of the anaerobic and facultative actinomycetes. Although certain methods of fermentative biochemistry have given greater insight into the physiology and metabolism of the members of the genus *Actinomyces*, the taxonomic problem of classifying new isolates rested primarily upon classical characteristics such as colony morphology, sugar fermentations, and physiological tests. In comparative growth and physiological studies, Howell and Pine (12, 17) made no attempt to designate species, although some were recognized as being strains of *A. israelii*. These strains and additional isolates were later classified by Howell et al. (11) as *A. israelii* or *A. naeslundii*. One strain, 296, was classified as *A. israelii* by these workers. However, it had certain of the characteristics of *A. naeslundii* and was so considered by Buchanan and Pine (1), although Howell (*personal communication*) was of the opinion that it was an atypical strain of *A. israelii*. In their studies of strains of *A. bovis*, Pine, Howell, and Watson (18) delineated three types of colonies which could serve in the majority of the cases to place a strain into the *A. bovis*, *A. naeslundii*, or *A. israelii* "group." However, one strain of *A. bovis* had the colony characteristics of *A. israelii*. Thus, for strains which could not be clearly identified as to species, an investigator was required to evaluate all the descriptive data, since no one criterion would serve to delineate clearly one species from the other.

However, the initial discovery by Cummins and Harris (5) of the general use of cell wall analyses for taxonomic identification and the extended application of such analyses by Cummins and Harris (6) to members of the genus *Actinomyces* emphasized that this method was most probably the only reliable method of identifying dubious strains. During this time, emphasis was also being placed on the comparative cell wall composition of microorganisms in relation to cell morphology (14).

The work reported here was initiated to answer the following questions: Within the three species mentioned above, was there a common chemical composition of their cell walls which related the mycelial form of *A. bovis* to the mycelial form in other species? What was the variation in chemical composition of the cell walls with variation in morphology? The results are discussed from the standpoint of taxonomic relationships among the species of *A. bovis*, *A. israelii*, and *A. naeslundii*.

MATERIALS AND METHODS

The following cultures of *Actinomyces* species were used: strain 279 *A. naeslundii*, isolated from a sinus following a tooth extraction, was obtained from L. Thompson; strain 263 *A. israelli*, isolated from a case of cervico-facial actinomycosis, was from the collection of T. Rosebury; strain 295 *A. israelii*, isolated from human pleural fluid by C. W. Emmons, was obtained from the American Type Culture Collection (*A. bovis* 10048); strain 296 *A. israelii*, isolated by C. W. Emmons from the left ankle of a patient with an atypical case of actinomycosis, was obtained from the American Type Culture Collection (*A. bovis* 10049); *A. bovis* strain P2R and P2S were isolated from a single case of lumpy jaw in a cow; *A. bovis* strains 13R and 13S were received from L. Georg; and strain 308 *Lactobacillus bifidus* was obtained from P. György.

Media used were the casein hydrolysate medium of Howell and Pine (12), the Casitone medium of Pine and Watson (19), Fluid Thioglycollate Medium (BBL) with 0.7% agar, and the *Actinomyces* maintenance medium (BBL). The methods of growth aerobically or anaerobically with and without CO_2 were those of Pine and Howell (17). Cultures of *A. bovis* spider-form or smooth-form were grown in 4-liter amounts of *Actinomyces* maintenance medium incubated at 37 C for 2 to 7 days under Na_2CO_3-pyrogallol seals. The inoculum consisted of 200 ml of *Actinomyces* maintenance broth culture showing typical morphology of the variant used. Before the cells were harvested, the cultures were checked by microscopic observation and preparation of streak plates to make certain that the spider variant had not produced the smooth variant or vice versa. Other growth procedures are given specifically in the text.

Acetone powders prepared from the cells were washed with ethyl ether, were air-dried to remove the residual solvent, and were then stored in a vacuum desiccator over anhydrous $CaCl_2$. For cell wall analyses, 100 mg of cells was suspended in water and was subjected to sonic disintegration with an instrument operating at 22 kc and 75 w, until completely disrupted. The particulate material was washed with distilled water several times by alternate centrifugation and resuspension. Hydrolysates were then prepared according to the procedure of Cummins and Harris (5), deleting the digestions with ribonuclease. Half of the samples were used for sugar analysis and half for amino acid analysis.

Amino acids were separated by two dimensional ascending chromatography through use of 31 by 33 cm sheets of Whatman no. 1 chromatography paper. The solvents were phenol-water (70:30, w/v) in an ammonia atmosphere (0.3% NH_4OH in water, by volume) in the first direction, and lutidine-water (65:35, v/v) in the second. Separation of hexosamine, muramic acid, lysine, ornithine, and arginine was dependent upon the relative "abundance" of the NH_3 atmosphere, with phenol-water as the primary solvent. In our system, the solvents were added to the bottom of the tank, the papers were hung immediately, and 0.3% ammonium hydroxide solution was added to a glass trough supported between the wires from which the papers were suspended. Under our conditions, glucosamine ran to the left of aspartic acid, muramic to the left of alanine, and arginine as an elongated spot in the upper right, whereas lysine and ornithine formed an elongated spot to the right of aspartic, extending upward and parallel to alanine. Different volumes of 6 N HCl-hydrolysates, from 50 mg (dry weight) of cells, were spotted on chromatography paper. Suitable amounts were then chosen, so that the spots obtained with ninhydrin were adequate for quantitative analysis. The amino acid spots were then analyzed quantitatively by the procedure of Kay, Harris, and Entenman (13). Sugars were separated by two dimensional ascending chromatography as above by use of phenol-water without the ammonia atmosphere in the first direction and butanol-pyridine-water (60:40:30, v/v) in the second. Sugars were eluted from the paper and were determined quantitatively by the method of Pridham (20). Glucosamine was identified by the formation of arabinose upon treatment with ninhydrin, the arabinose being identified by paper chromatography (23); hexosamines were determined quantitatively on cell wall hydrolysates by the Rondle and Morgan procedure (21). Sugars, in cell wall hydrolysates, were determined by the phenol-sulfuric acid method of Dubois et al. (9). This procedure does not determine hexosamine; consequently it was used to determine nonamino sugars directly. Glucose was determined by the use of Glucostat (Worthington Biochemical Corp., Freehold, N.J.). Reducing sugar was determined by the procedure of Park and Johnson (15), methyl pentoses were determined by the procedure of Dische and Shettles (8), and amino acids by the procedure of Troll and Cannan (25).

RESULTS

Studies on the factors influencing the cell morphology of A. israelii 296. Strains 296 and 279 grew anaerobically in the presence of carbon dioxide as branched rods with some elongated cells. Cells grown in the presence of air with an increased carbon dioxide concentration became "diphtheroid" and were shorter, rounder, and less branched. To determine morphological variation with changes of media, experiments were conducted using casein hydrolysate medium, thioglycolate broth, and aerobic and anaerobic conditions. The two strains grew with essentially identical morphology under the various conditions; it was also observed that growth of both strains within 24 hr on Fluid Thioglycollate Medium was exceedingly filamentous. Clumps of the original cells (Fig. 1a), when transferred from the casein hydrolysate medium to Fluid Thioglycollate Medium, were surrounded within 24 to 48 hr with long streamers of filaments. These cell aggregations presented a gross morphology described as "Medusa heads" (Fig. 1b and 1c). Subsequently, these filaments changed to small diphtheroidal elements which were smaller than the cells of the original inoculum (Fig. 1d). This

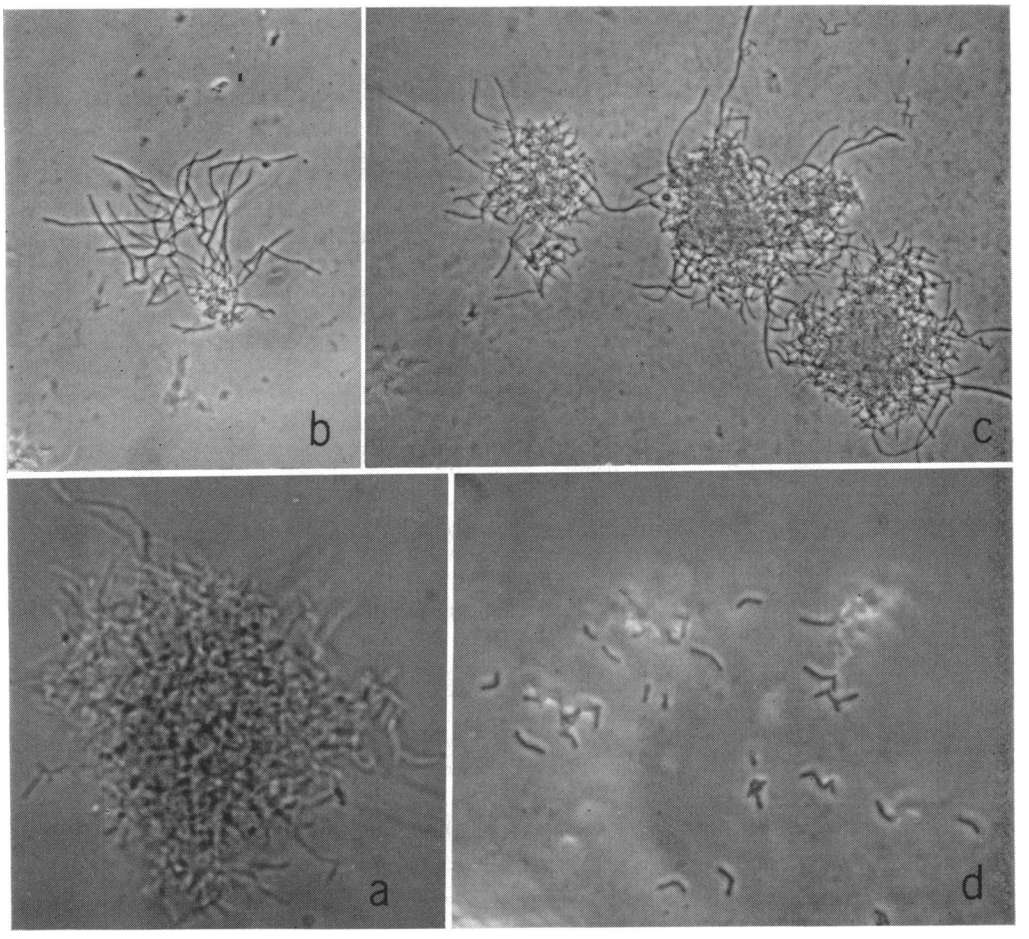

FIG. 1. *Actinomyces israelii 296. (a) Cells of a 48-hr culture in Actinomyces maintenance broth. Medium dark phase. 970 ×. (b) Early stage of filamentous cells from 48-hr culture in thioglycolate broth, transferred from Actinomyces maintenance broth. Medium dark phase. 200 ×. (c) As (b), different field, showing "Medusa heads." Medium dark phase. 200 ×. (d) Cells from a 3-day culture in thioglycolate broth. Medium dark phase. 970 ×.*

effect of the thioglycolate medium was tested on strains *A. israelii* 263 and 295 and *L. bifidus* 308. An intermediary effect was observed on strains 295 and 308, for the cells were somewhat more elongated; branching cells were observed initially, but long filamentous forms were not observed. The thioglycolate broth did not affect the morphology of strain 263. The morphology was not significantly different, whether the tube was incubated anaerobically, i.e., with the pyrogallol-carbonate and pyrogallol-KOH seals, or aerobically without any seal.

The long filamentous nature of some of the cells in the thioglycolate broth recalled the results of Webb (26, 27), who observed that a magnesium deficiency in *Clostridium welchii* induced the formation of long nonbranching filaments. By addition of the components of the casein hydrolysate medium individually or in groups to Fluid Thioglycollate Medium, it was found that only the addition of KH_2PO_4, cysteine, or glutathione would prevent the formation of filaments. No other components, such as trace metals or growth factors of the casein hydrolysate medium, influenced filament formation.

Hot-cold incubation periods of the inoculum or aging in a refrigerator at 5 C did not cause filament formation. Since filament formation was obtained only on the first transfer to the new medium, it was concluded that the transfer of the culture from casein hydrolysate medium to the thioglycolate broth caused a temporary metabolic imbalance due to the deficiency of those components mentioned above.

Effect of morphology on cell wall composition. Experiments were then conducted to determine whether there was any significant change in cell wall composition which could be related to changes in morphology as induced by changes in medium. To grow large volumes of cells, certain changes were made in the media used. First, the *Actinomyces* maintenance medium was used since it was commercially available, easily prepared, and gave excellent filament formation when the cells were first grown on this medium and then transferred to the thioglycolate medium. The thioglycolate broth was prepared without agar. Four cultures, 4 liters each, of strain 296 were made; one culture was incubated 7 days on the *Actinomyces* maintenance medium, a second culture was incubated 3 days on the thioglycolate broth, and a third culture was incubated 7 days on the thioglycolate broth. The inoculum for each of these three cultures was 60 ml of a 3-day-old culture grown on *Actinomyces* maintenance broth. Cells of the first culture are shown in Fig. 1a; those of the second, which are also typical of the third culture, are shown in Fig. 1d. To obtain cultures that most resemble the stage of cellular morphology depicted in Fig. 1c, 4 liters of the *Actinomyces* maintenance medium was prepared and inoculated with 60 ml of the 48-hr culture grown in the same medium. The large flask was sealed with a pyrogallol-sodium carbonate seal and was incubated 3 days at 37 C. The flask was then placed at 4 C for 24 hr, during which time the cells settled to the bottom. The flask was then removed, and the supernatant fluid was withdrawn aseptically. The flask was refilled with sterile thioglycolate broth. After 16 hr at 37 C, a sample of the cell suspension was withdrawn and observed with the phase microscope. Although the cell clumps were more filamentous than those shown in Fig. 1a, there were no "Medusa heads" or filaments typical of those shown in Fig. 1b and 1c. At 20 hr, the cell clumps were decidedly more filamentous than those depicted in Fig. 1a and had several extended hyphae. Nevertheless, at this time, the temperature of the culture was placed at 4 C. It was assumed that further mycelial growth would occur during the several hours required for significant cooling. The flask was harvested for cell wall preparations the following day.

Because of the taxonomic interest in the relationship of strain 296 to *A. naeslundii*, a 4-liter culture of *A. naeslundii* strain 279 was grown on the *Actinomyces* maintenance medium, and cell wall analyses of the two organisms were conducted concomitantly.

The results of the quantitative amino acid analyses are given in Table 1 with calculated molar concentrations of amino acids relative to alanine.

TABLE 1. *Analyses of strains of Actinomyces cell wall amino acids, in terms of relative moles in cells of different morphology*

Amino acids	*A. israelii* 296								*A. naeslundii*	
	AM[a] for 7 days (mycelial)		AM-Thio[a] for 20 hr ("Medusa head")		Thio for 3 days (diphtheroid)		Thio for 7 days (diphtheroid)		AM for 7 days (diphtheroid)	
	μmoles	Rel. mole[b]	μmoles	Rel. μmole	μmoles	Rel. mole	μmoles	Rel. mole	μmoles	Rel. mole
Aspartic	9.8	1.1 (1)	11.3	1.1 (1)	5.0	0.5 (1)	5.8	0.6 (1)	2.2	0.4
Glutamic	52.8	6.0 (6)	41.8	4.3 (4)	29.5	2.9 (3)	26.1	2.6 (3)	32.2	5.2 (5)
Alanine	35.2	4.0 (4)	39.4	4.0 (4)	40.5	4.0 (4)	40.1	4.0 (4)	24.7	4.0 (4)
Lysine plus ornithine[c]	6.9	0.8 (1)	22.6	2.3 (2)	19.9	2.0 (2)	18.2	1.8 (2)	4.0	0.6 (1)
Hexosamine	3.7	4.2 (4)	49.7	5.1 (5)	71.3	7.0 (7)	66.2	6.6 (7)	7.8	1.3 (1)
Valine	10.6	1.2 (1)	10.3	1.0 (1)	5.1	0.5 (1)	7.7	0.8 (1)	1.2	0.2
Comb. 4[c]	15.0	1.7 (2)	19.9	2.0 (2)	9.4	0.9 (1)	10.8	1.1 (1)	11.8	1.9 (2)
Threonine	4.2	0.5	7.7	0.8 (1)	3.9	0.4	3.2	0.3	0.0	0.0
Muramic	1.8	0.2	5.7	0.6	4.3	0.4	2.9	0.3	4.6	0.7 (1)
Serine	0.0	0.0	0.0	0.0	9.3	0.9 (1)	10.3	1.0 (1)	0.0	0.0
Arginine	4.7	0.5	5.8	0.6	0.0	0.0	0.0	0.0	0.3	0.0
Glycine	4.4	0.5	4.9	0.5	10.0	1.0 (1)	10.0	1.0 (1)	2.5	0.4

[a] AM = *Actinomyces* maintenance medium, and Thio = thioglycolate medium.

[b] Rel. mole = molar concentration relative to alanine with a value of 4. Rounded whole-number molar values are given in parentheses.

[c] The solvents used in chromatographic analysis of the amino acids did not separate lysine from ornithine. These two amino acids were therefore determined together as were methionine, leucine, isoleucine, and phenylalanine which are listed as comb. 4.

Although a virtually complete spectrum of amino acids was observed on the paper chromatograms, for strain 296 there were six major cell wall constituents of interest: glutamic acid, alanine, lysine, glucosamine, aspartic acid, and valine. With the transfer of strain 296 from the *Actinomyces* medium to the thioglycolate broth, no major qualitative change took place in the amino acids present. Quantitatively, however, a shift took place from an approximate ratio of aspartic-glutamic-alanine-lysine concentrations of 1:6:4:1 to that of 1:4:4:2 in the 20-hr thioglycolate culture and to that of 0.5:3:4:2 at the end of 7 days. Aspartic acid was present in significant amounts in all samples. The amino acid analyses of *A. naeslundii* showed essentially the identical qualitative amino acid picture as strain 296 grown on the *Actinomyces* maintenance medium.

Throughout the analyses of the amino acid chromatograms of strain 296, a spot, either glycine or asparagine, was obvious by its difference in color. By use of ^{14}C-glycine alone or of mixtures with asparagine or with the cell wall preparations or both, and by variation of the ammonium atmosphere, this spot was identified as glycine. The relative molar ratios of glycine in the cell wall preparations were therefore determined in an additional series of experiments and are given in Table 1.

The results of the sugar analyses are given in Tables 2 and 3. In general, galactose and glucosamine were the two major constituents of strain 296. Although mannose, rhamnose, and arabinose were present, there was no consistent pattern with these sugars. There was a temporary increase in the rhamnose to galactose ratio during the "Medusa head" stage of growth to 6:10; this dropped in the next stage to the more characteristic ratio of 1:10. Hexosamine was always present in large concentrations, and direct quantitative analyses of the hydrolysates after $BaSO_4$ precipitation suggested a definite increase in hexosamine in the 3- and 7-day cells grown in thioglycolate (Table 3). Of the secondary sugars, only rhamnose appeared to be consistently present. Although the analyses of amino acids of the two strains did not qualitatively distinguish them, the sugar analyses clearly differentiate *A. naeslundii* strain 279 from strain 296. Strain 296, by virtue of having only galactose as the major neutral carbohydrate component of its cell wall, must be classified as a strain of *A. israelii* (2). *A. naeslundii* is easily characterized by relatively equal concentrations of glucose, fucose, rhamnose, and 2-deoxytalose (Table 2). Small but significant amounts

TABLE 3. *Effect of media on relative hexosamine-sugar ratios in Actinomyces israelii 296[a] cell walls*

Medium[c]	Culture age	Hexosamine[b]	Sugars[b]	Hexosamine/sugar
		mg	*mg*	
AM	7 days	13.4	19.7	0.68
AM-Thio	20 hr	3.2	4.7	0.68
Thio	3 days	11.2	9.2	1.22
Thio	7 days	15.1	18.5	0.82

[a] Digests with 2 N H_2SO_4 of purified cell walls were neutralized with $Ba(OH)_2$, filtered, and concentrated.

[b] Hexosamine was determined by the Rondle and Morgan (21) procedure; and sugars were determined by the phenol-sulfuric acid procedure of Dubois et al. (9).

[c] AM = *Actinomyces* maintenance medium, and Thio = the thioglycolate medium.

TABLE 2. *Analyses of Actinomyces cell wall sugars, in terms of relative moles in cells of different morphology*

Sugar	*A. israelii*								*A. naeslundii*	
	AM[a] for 7 days (mycelial)		AM-Thio[a] for 20 hr ("Medusa head")		Thio for 3 days (diphtheroid)		Thio for 7 days (diphtheroid)		AM for 7 days (diphtheroid)	
	μmoles	Rel. mole[b]	μmoles	Rel. mole	μmoles	Rel. mole	μmoles	Rel. mole	μmoles	Rel. mole
Glucose	+	−	+	−	+	−	+	−	21.7	2
Fucose	−	−	−	−	−	−	−	−	31.7	3
Rhamnose	1.7	0.3	2.6	6.2	0.3	1.0	3.1	1.5	40.8	4
2-Deoxytalose	−	−	−	−	−	−	−	−	31.7	3
Mannose	9.4	9.0	−	−	−	−	7.2	3.4	3.0	4
Galactose	43.4	10.0	4.2	10.0	31.1	10	21.1	10.0	−	−
Arabinose	0.6	−	−	−		−	3.1	−	+	−

[a] AM = *Actinomyces* maintenance medium, and Thio = the thioglycolate medium.

[b] Rel. mole = molar concentrations relative to galactose with a value of 10.

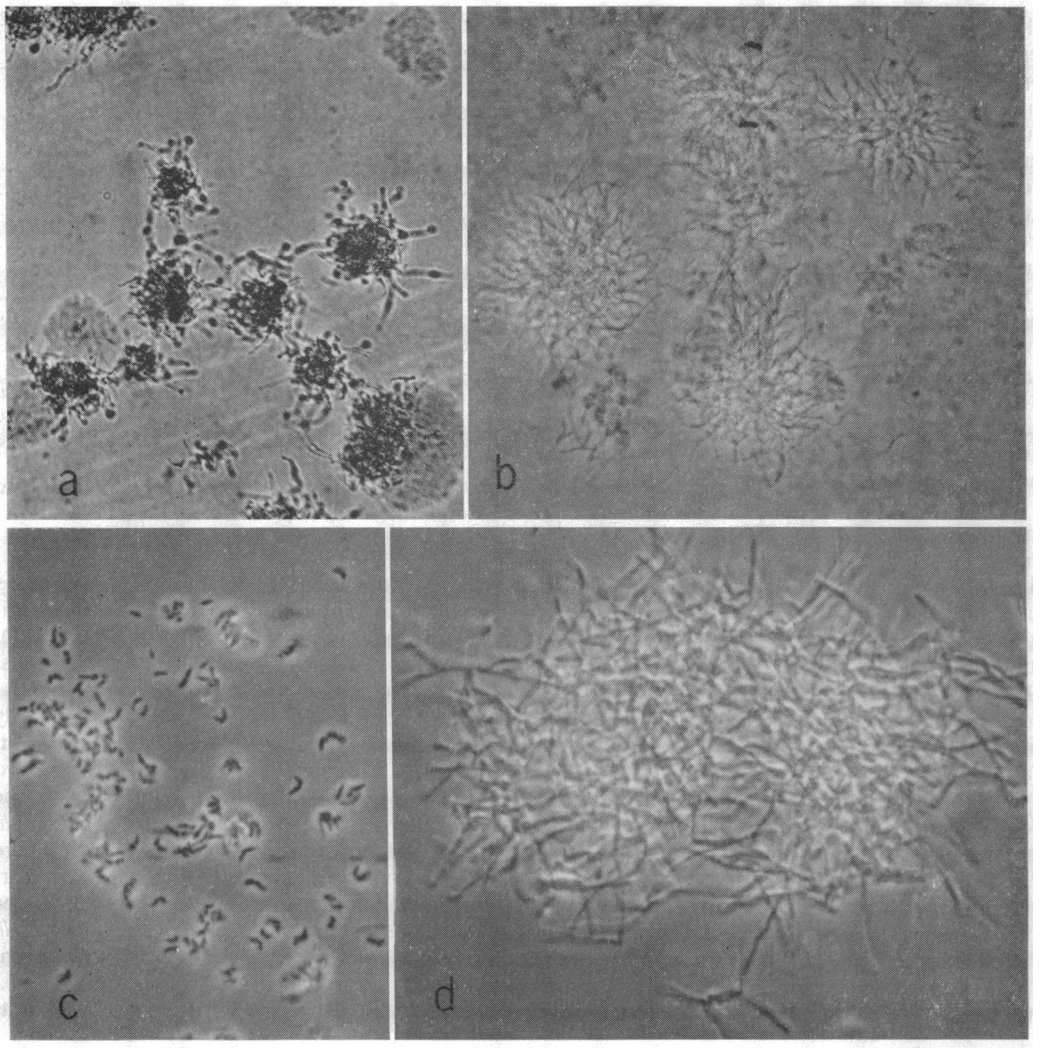

FIG. 2. *Actinomyces bovis.* (*a*) *Colonies of P2R (spider form) and P2S (smooth) on original isolation plate 290 ×.* (*b*) *Colonies of P2R (spider form) grown from inoculum used to inoculate 4L culture.* (*c*) *Strain P2S (smooth) 48-hr culture in Actinomyces maintenance broth. Medium dark phase. 970 ×.* (*d*) *Strain 13R. Cells of the 4L culture in Actinomyces maintenance broth, 48 hr. Medium dark phase. 970 ×.*

of mannose are also present. Galactose was not present in significant amounts.

Comparative colony and cellular morphology of A. bovis strains. Colony and cellular morphology are adequately depicted in Fig. 2a and b. We emphasize that many difficulties were encountered in obtaining and maintaining pure cultures of the spider form. We were originally able to isolate and maintain the spider form for several years; during this period, it never reverted to the smooth form in vitro. However, after several years, P2R and 13R spontaneously gave rise to the smooth variant, and it finally became impossible to maintain these strains in their pure mycelial form. Similar difficulties were described by Georg, Robertstad, and Brinkman (10). All analyses reported here were made on cultures of pure spider form (Fig. 2b and d), obtained either fortuitously or at that earlier time when the cultures were not undergoing morphological variation.

Cell wall analyses of A. bovis spider forms P2R and 13R and smooth forms P2S and 13S. Table 4 shows the comparative analyses of cell walls after

hydrolysis with 2 N HCl for 2 hr. This method of hydrolysis was used, since it was not completely destructive to sugars and since a relative comparison of sugar and amino acids could be made. No significant differences were found between the individual strains in regard to the ratio of amino acid to reducing sugar, this ratio being between 0.45 and 0.50. However, the ratio of hexosamine to methyl pentose in the spider-form strains, P2R and 13R, was approximately three times that found in the smooth variants. This apparent increase in hexosamine was further reflected by the increased ratio of hexosamine to amino acid and the decreased ratio of methyl pentose to reducing sugar in the spider forms (Table 4).

The cell walls of all strains were then analyzed after hydrolysis by the standard procedures of Cummins and Harris (6). The results of the quantitative analyses of the individual amino acids and sugars released for all strains are given in Tables 5 and 6, respectively. The relative molar concentrations are also given in these tables, by use of alanine with a value of 4 and rhamnose with a value of 10. Of the amino acids observed on the paper chromatograms, only a few were present in sufficient amounts to be seen; this is contrary to the chromatograms of *A. israelii* and *A. naeslundii* in each of which a complete spectrum of amino acids was observed. Of the amino acids, only aspartic acid, glutamic acid, alanine, and lysine were present in major concentrations. Hexosamine was also present. Secondly, the spider forms contained an insignificant amount of aspartic acid. The molar concentrations of the remaining amino acids were the same in spider and smooth strains. Finally, hexosamine was higher in both spider strains, although the ratio of P2R hexosamine to P2S hexosamine was lower than that anticipated from the data of Table 4. Although there were minor differences among them, the relative molar concentrations of amino acids were alike in the four strains (with the above described differences) and were different from those ratios observed for the strains of *A. israelii* and *A. naeslundii*.

TABLE 4. *Chemical analyses of cell wall hydrolysates of spider form (R) and smooth (S) variants of Actinomyces bovis*[a]

Determinations	P2R	P2S	13R	13S
Reducing sugar[b], mg	2.68	1.85	2.12	0.85
Glucose, mg	0.13	0.14	0.13	0.06
Methyl pentose[b], mg	0.80	1.24	1.24	0.72
Hexosamine, mg	0.58	0.33	0.54	0.13
Amino acids, mg	1.26	0.91	1.07	0.38
Amino acid/reducing sugar	0.47	0.49	0.50	0.45
Hexosamine/methyl pentose	0.72	0.27	0.44	0.18
Hexosamine/amino acid	0.46	0.36	0.50	0.34
Methyl pentose/ reducing sugar	0.30	0.67	0.59	0.85

[a] A 17-mg amount of whole dried cells was treated with trypsin and pepsin and was then acid-hydrolyzed in 2 N HCl for 2 hr in boiling water bath.

[b] Rhamnose as a standard.

Although there were no qualitative sugar differences between the spider and smooth forms, there were differences in the molar concentrations of the sugars between the respective smooth and spider forms. In particular, there appeared to be a marked increase in 2-deoxytalose in the shift from the spider to the smooth form. In addition, strains P2R and P2S appeared to have only trace amounts of fucose, whereas this sugar was present in strains 13R and 13S.

TABLE 5. *Cell wall analyses of amino acids of Actinomyces bovis spider (R) and smooth (S) forms*

Amino acids	13R		13S		P2R		P2S	
	μmoles	Rel. mole[a]	μmole	Rel. mole	μmoles	Rel. mole	μmoles	Rel. mole
Aspartic acid	0	0	0.735	3	0.050	0	0.110	3
Glutamic acid	0.152	2	0.396	2	0.641	3	0.097	3
Alanine	0.337	4	1.12	4	0.937	4	0.149	4
Lysine	0.257	3	0.930	3	0.633	3	0.156	4
Muramic acid	0.014	0.2	0.048	0.2	0.048	0.2	0.020	0.1
Hexosamine	1.15	14	0.964	3	1.36	6	0.178	5
Arginine	0	0	0.014	0	0.019	0	0.006	0
Valine	0.073	1	0.029	0	0.035	0	0.014	0
Comb. 4[b]	0.088	1	0.075	0	0.032	0	0.024	1

[a] Rel. mole = molar concentrations relative to alanine with a value of 4.

[b] See Table 1, footnote *c*.

TABLE 6. *Cell wall analyses of sugars of Actinomyces bovis spider (R) and smooth (S) forms*

Sugars	13R		13S		P2R		P2S	
	μmoles	Rel. mole[a]	μmoles	Rel. mole	μmole	Rel mole	μmoles	Rel. mole
Glucose	0.314	3	0.311	3	0.136	3	0.606	5
Mannose	0.289	3	0.168	2	0.136	3	0.344	3
Fucose	0.397	4	0.324	3	0	0	0.073	0.5
Rhamnose	1.073	10	1.173	10	0.453	10	1.295	10
2-Deoxytalose	0.344	3	1.675	17	0.117	3	1.229	9

[a] Rel. mole = molar concentrations relative to rhamnose with a value of 10.

DISCUSSION

Of the various species considered as members of *Actinomycetales*, relatively few are reported as having aspartic acid in the cell wall (2, 3, 6, 28). Of those which do have aspartic acid, with the exception of *A. propionicus* (17) none forms mycelial colonies. Consequently, the finding that the spider form of *A. bovis* did not contain aspartic acid suggested that perhaps a common relationship of cell wall composition to morphology might be found. It is apparent from the results reported here and from a closer examination of other exceptions within the order that there is no common denominator relating cell wall composition and morphology within the order and within the genus *Actinomyces* itself. In the species *A. bovis*, the spider form compared to the smooth form showed a gain in hexosamine and also showed the complete loss of aspartic acid; in the smooth form, an aspartic acid to alanine ratio of 3:4 was found. Comparing the chemical composition of the cell walls of *A. israelii* 296 under the various conditions of growth and morphology, there was a decrease in the aspartic acid to alanine ratio from 1:4 to 0.5:4 as the morphology changed from an interwoven mycelium to a small diphtheroidal cell. More striking in this species was the decrease in the glutamic acid to alanine ratio (6:4 to 3:4) in the "Medusa head" form. The ratio of aspartate to alanine in *A. naeslundii* was only 0.4:4.0, but the culture was essentially diphtheroid. Thus, among species which at one time appeared closely related, there was no mutual relation relative to chemical composition and morphological change. The absence of aspartic acid does not "dictate" a mycelial morphology in all the species.

Initially, *A. israelii* and *A. bovis* were isolated, identified and characterized primarily by the diseases they caused, their colony characteristics, and their cellular morphology. At one time, these two species were not accepted by all as being separate taxons, and there is little doubt that much of the confusion was related to the various colony and cellular types produced by them (22).

Of the present characteristics used for the identification of species of *Actinomyces*, none has shown the promise of taxonomic specificity of the cell wall analyses initially described by Cummins and Harris (6, 7). In all details, our results are in agreement with those reported by these workers (2–7). Cummins (4) reported that *A. israelii* strains (and those of *A. naeslundii, personal communication*) contained ornithine; *A. bovis* strains do not. Repeating their experiments, we have confirmed their results with the above strains, and valid distinction of these species can be made by their procedure. Qualitatively as seen on the chromatograms, both species have very little aspartic acid. However, we would like to point out that, quantitatively, aspartic acid should not be dismissed as a "minor" amino acid in the cell wall mucopeptide of *A. israelii* and *A. naeslundii*, since it was present in the same order of magnitude as lysine (or lysine + ornithine). Similarly, valine and glycine must be considered as important constituents of the cell wall mucopeptide, since they and aspartic acid are consistently present in greater concentrations than the remaining amino acids.

Attention is also directed to the close similarity of the cell wall composition of *A. naeslundii* and the *A. bovis* strains. Both species contain major concentrations of glucose, mannose, fucose, rhamnose, 2-deoxytalose, glutamic acid, alanine, and lysine. The spider forms of *A. bovis* contain small if not neglible amounts of aspartic acid and could be, therefore, readily confused with *A. naeslundii*, because the two dimensional chromatographic system employed above does not readily discern the ornithine + lysine of *A. naeslundii* from the lysine of the spider form of *A. bovis*. The smooth forms of *A. bovis* would be readily identified by their high aspartic acid content.

In our earlier work (17), it appeared that the use of oxygen for growth with increased cell yields separated strains of *A. naeslundii* from those of *A. israelii* since a major metabolic difference was indicated. Strain 296 was chosen for further study, since it presented numerous characteristics com-

mon to both species. In the presence of oxygen, this strain not only showed continous growth as did strains of *A. naeslundii* but actively utilized oxygen to double its cell yields; concomitantly, glucose was oxidized to acetate and CO_2 (1, 1*a*). Consequently, Pine considered this strain a strain of *A. naeslundii* (1, 1*a*). With the results obtained on the composition of its cell wall, it is quite clear that this strain must be classified as a strain of *A. israelii*. It is equally clear that, in the sense of utilization of oxygen for growth, both *A. naeslundii* and *A. israelii* must be considered as potentially facultative organisms. However, all strains of *A. israelii* cannot grow in shake cultures with continuous transfers in air plus CO_2, or, if they do, they may require unknown growth factors (1).

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