CLASSIFICATION AND PHYLOGENETIC RELATIONSHIP
OF MICROAEROPHILIC ACTINOMYCETES

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ABSTRACT. In consideration of the genera proposed for the family Actinomycetaceae, weighted considerations were given to the factors of morphology, carbon dioxide and oxygen utilization, anaerobic and aerobic fermentation balances, glucose metabolism, presence of catalase and haemoproteins, and cell wall composition. Primarily, on the basis of cell wall composition but strongly supported by metabolic and fermentation characteristics, it was concluded the genus Arachnia belongs in the family Propionibacteriaceae. Similarly, the genus Bacterionema is more suitable within the family Mycobacteriaceae or Corynebacteriaceae. The family Actinomycetaceae would retain Actinomyces israelii, A. bovis, A. naeslundii, A. odontolyticus, A. viscosus, and Rothia dentocariosa.

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This paper reviews certain studies pertinent to the classification of microaerophilic actinomycetes and the establishment of phylogenetic relationships within the species. I have attempted to evaluate each of the studies in relation to the taxonomic criteria used previously by Pine and Georg (1964). Such evaluations permit a "weighted" approach to the establishment of phylogenetic relationships; perhaps the assimilated data will provide answers to questions of classification which are more satisfactory not only to me but to others who are concerned with the group of organisms comprised primarily of anaerobic and facultative actinomycetes. No attempt is made to review all the literature, and several excellent papers, particularly in the area of serological investigations, are not included. Other papers are listed in the references, although they are not specifically cited in the text.

Organisms of the family Actinomycetaceae.

Buchanan and Pine (1962) suggested a phylogenetic progression in the order Actinomycetales which was based on the presence or absence of true mycelium, the presence or absence of catalase, fermentation balances, and differentiation of lysine or diaminopimelic acid (DAP) in the cell walls. In a report to the Committee on Taxonomy of the Microaerophilic Actinomycetes, Pine and Georg (1964) incorporated

newly described genera into an expanded classification scheme and emphasized certain problems pertinent to the members of a redefined family Actinomycetaceae. Several of the problems relating to nomenclature have since been corrected in publications which more clearly describe a given taxon and present changes in the generic designation. Through the efforts of the Committee under the chairmanship of John M. Slack, the family which has been presented for consideration has been expanded to contain five genera and 10 species (Table 1). With increased knowledge about these organisms comes the recognition that the family Actinomycetaceae as presented is complex. Indeed its members exhibit essentially a complete spectrum of anaerobic to aerobic metabolism, three types of fermentation, and diverse cell wall compositions. There is, however, substantial reason to suspect mutual relationships among these characteristics, and we will further examine certain of them.

Morphology: cellular, microcolony and macrocolony.

The various species of the family as proposed are related in cell morphology if not in microcolony structure. There is little to differentiate the various members of the genus Actinomyces on the basis of cell morphology as seen with the light microscope. All of the organisms have the potential of having lateral points of growth in addition to the polar growing tips generally observed in the unbranched bacteria. With the electron microscope certain differences between A. bovis and A. naeslundii, Arachnia propionica, (Overman and Pine, 1963) and Bifidobacterium bifidum become apparent. In its typical cell morphology A. bovis is more round; its reproduction is a budding type of growth different from the remaining species which are rod shaped and whose reproduction is by elongation from a growing tip. Cell walls of A. bovis and Ar. propionica are 1/2 to 1/3 of the thickness of other species. The great tendency for strains of Ar. propionica and Rothia dentocariosa to form spheroplasts and of R. dentocariosa to form coccoid cells distinguishes them from other species (Buchanan and Pine, 1962) (Gerencser and Slack, 1967) (Brown, Georg, and Waters, 1969), but nothing about this characteristic suggests phylogenetic relationships. Spheroplast formation is easily observed in old cultures of A. bovis (Pine, unpublished) and spheroplast formation is apparent from the photographs of A. odontolyticus (Batty, 1958). However, the morphology and process of cell wall multiplication of Bacterionema matruchotii as depicted by Gilmour (1961) are characteristic of this species only. Observations made in our laboratory with the Gram stain, modified acid fast stain, and phase optics strongly suggested that a somewhat "higher order of reproduction" or survival by a primordial spore was present (Pine and Watson, unpublished results). The suggestion remains to be verified by more extensive studies, however.

In their comparative study of A. bovis and A. israelii, Pine, Howell, and Watson (1960) emphasized the characteristic microcolony differences between A. bovis, which generally did not demonstrate the spreading "spider" colony; A. israelii, which did; and A. naeslundii which produced a spider colony only in the very early stages of growth followed by dense cell reproduction in the center. With the exception of a single mycelial mutant (P2R), no strain of A. bovis formed a true mycelium in vitro. Ultimately, by inoculation into the hamster, true mycelium

Table 1. Genera and species proposed for the family Actinomycetaceae.

Organism	Synonym	Selected References
I. Genus <u>Actinomyces</u>		
1. <u>A. bovis</u>	-	Harz (1879); Silberschmidt (1900, 1901); Erikson (1940); Thompson (1950); Pine, Howell and Watson (1969); Cummins (1962).
2. <u>A. israelii</u>	-	Wolff and Israel (1891); Thompson (1950); Howell, Murphy, Paul and Stephan (1959); Cummins (1962); Slack, Landfried and Gerencser (1969).
3. <u>A. naeslundii</u>	-	Thompson and Lovestedt (1951); Howell, Murphy, Paul and Stephan (1959); Pine and Boone (1968).
4. <u>A. viscosus</u>	(hamster organism" <u>Odontomyces viscosus</u>	Howell and Jordan (1963); Howell (1963); Georg, Pine and Gerencser (1969).
5. <u>A. eriksonii</u>	-	Georg, Robertstad, Brinkman and Hicklin (1965).
6. <u>A. odontolyticus</u>	-	Batty (1958).
II. Genus: <u>Rothia</u>		
1. <u>R. dentocariosa</u>	<u>Nocardia salivae</u>	Onisi (1949); Davis and Freer (1960); Roth and Thurn (1962); Brown, Georg and Waters (1969).
III. Genus: <u>Arachnia</u>		
1. <u>Ar. propionica</u>	<u>Actinomyces propionicus</u>	Buchanan and Pine (1962); Gerencser and Slack (1967); Pine and Georg (1969).
IV. Genus: <u>Bifidobacterium</u>		
1. <u>B. bifidum</u>	<u>Lactobacillus bifidus</u> <u>Actinomyces parabifidus</u> <u>Lactobacillus parabifidus</u> <u>Actinobacterium bifidum</u>	Prévot (1961); Pine and Georg (1965); Puntoni (1937); Weiss and Rettger (1938a, b).
V. Genus: <u>Bacterionema</u>		
1. <u>Bact. matruchotii</u>	<u>Leptotrichia</u>	Gilmour, Howell and Bibby (1961); Gilmour and Beck (1961); Gilmour (1961); Howell and Pine (1961).

was observed in vivo but only with the consequential recognition that mycelium formation was of a limited taxonomic value. Erikson (1949) had described this characteristically smooth colony of A. bovis much earlier. From the standpoint of identification, however, the microcolony serves to delineate several of the species (Howell et al., 1959; Georg, Robertstad, Brinkman, and Hicklin, 1964). These results are summarized in Table 2.

Description of physiological characteristics of certain of the species.

In a comprehensive review, Rosebury (1944) described the physiological characteristics of Actinomyces strains which undoubtedly represented both A. israelii and A. naeslundii. In a general way, I wish to emphasize certain of these characteristics in the light of more recent knowledge. The factors with which I am concerned are (a) the significance of rates of growth, (b) the effect of carbon dioxide, of medium, and oxygen on growth and (c) the need for unknown factors.

Based on a medium described by Kline and Barker (1950), Howell and Pine (1956) developed a casein hydrolysate and synthetic medium that maintained the growth of Actinomyces strains which were later recognized as being both A. israelii and A. naeslundii; 2 strains (bovine) (278 and 284), which had been obtained from the collection of Thompson and which in retrospect were mycelial mutants similar to strain P2R (Pine, Howell, and Watson, 1960), failed to grow after several transfers and were lost. The strains tested showed three characteristic types of growth. One type of growth (Represented by strains 279 and 286, A. naeslundii) was extremely rapid; growth reached its maximum within 24 to 48 hrs, and the cells were smoothly dispersed. A second type of growth (represented by strains 277 and 287, A. israelii) was extremely slow; growth reached a maximum within 5 to 7 days, and the cell masses were so granular that homogenization with a Potter-Elvehjem homogenizer or by forced expulsion through a narrow bore pipette was necessary for accurate optical density measurements. Strains of a third type showed growth characteristics intermediate between these two extremes. The analyses of the fermentation products on either of these two media showed essentially a homolactic acid fermentation with sufficient formate to give a significantly high redox value. However, when the medium was fortified with casitone, the characteristic growth of several strains changed; granular growth became smooth, the cell yields appeared higher, and much less lactic acid was formed (Pine and Howell, 1956).

As a result of several experiments, certain points pertinent to carbon dioxide utilization became clear. That carbon dioxide stimulated the growth of Actinomyces (Rosebury, 1955) had been well known, and to account for this need and to give strict anaerobiosis, we had used the conventional pyrogallol-sodium carbonate seal. With the decrease of lactate formed in the presence of CO₂ and casitone, it became apparent that carbon dioxide was not needed on the growth factor level but that carbon dioxide was being metabolized at a substrate level with the formation of succinic acid (Pine and Howell, 1956). Later studies by Buchanan and Pine (1965) showed that carbon dioxide was also required for the synthesis of aspartic acid for which Actinomyces had no permease. In comparisons of the need for, and mechanism of, carbon dioxide fixation by strains of L. bifidus and Actinomyces,

Table 2. Cellular and colonial morphological characteristics within the family Actinomycetaceae.

	Cell wall thickness	basic cell structure	special cell formed	Atypical cells, spheroplasts	microcolony
<i>Bifidobacterium bifidum</i>	200 A	rod-bifrucate	-	-	smooth, entire edge
<i>Actinomyces eriksonii</i>		rod-bifrucate	-	-	smooth, entire edge
<i>A. bovis</i>	100 A	spheroid-budding	-	few spheroplasts	smooth, entire edge "spider-mutant"
<i>A. israelii</i>	290 A	rod-branched	-	-	spider colony
<i>A. naeslundii</i>	200 A	rod-branched	-	-	spider colony
<i>A. odontolyticus</i>		rods-"diphtheroid"	-	spheroplasts "atypical" cells	smooth, entire edge
<i>A. viscosus</i>		elongated hyphae, branching	-	-	mycelial colony
<i>Rothia dentocariosa</i>		elongated hyphae, branching	coccoid cells	-	mycelial colony
<i>Arachnia propionica</i>	114 A	rods-branched	-	spheroplasts	spider colony
<i>Bacterionema matruchotii</i>		elongated hyphae	"acid-fast" terminal "bacillus-like" body on clavate hyphae, or intercalary	-	mycelial colony

it became obvious that carbon dioxide had little effect upon the fermentation balances of L. bifidus, although carbon dioxide was required for growth (Pine and Howell, 1956) (Pine, 1956; 1960). These studies pointed out that the utilization of carbon dioxide by Actinomyces was unique and that carbon dioxide activated a series of reactions which affected cell yields and growth characteristics depending upon the medium used. In the presence of adequate carbon dioxide and casitone, Actinomyces fermented glucose with an increase of organic acid of organic acid of 30 to 100 percent over that obtained when the fermentation was homolactic.

Puntoni (1937) reported that strains of bifidobacterium were less sensitive to low pH than were Actinomyces. This observation was supported by the fact that the strains of L. bifidus fermented 86 to 89 percent of the glucose before the fermentation was stopped by the low pH (Pine and Howell, 1956).

Working on the basis of this information, Pine and Watson (1959) further modified and evaluated the casitone medium to develop an isolation and maintenance medium superior to any others tested. The medium also permitted excellent growth and maintenance of strains of Corynebacterium acnes, C. liquefaciens, L. bifidus, B. matruchotii (Leptotrichia buccalis), and four species of Actinomyces. To prevent the drop of pH accompanying high carbon dioxide and glucose utilization, the glucose concentration was reduced to 0.5 percent and 1.5 percent phosphate was used as a buffer. Casitone was used, since it was found to contain growth factors necessary for maintaining certain strains and was required to promote the aerobic growth of others. Uracil and coenzyme A were required for maximum growth of A. bovis; cysteine was added as a reducing agent and was later shown to be required for growth (Christie and Porteous, 1962; Buchanan and Pine, 1963); the presence of starch and an initial pH of 6.5 was considered mandatory in view of the inhibitory effect of higher pH's under anaerobic conditions in the absence of starch (Howell and Pine, 1956) and under aerobic conditions regardless of medium (Buchanan and Pine, 1963). Yeast extract was added for the "unknown". In a five-year clinical evaluation, this medium was superior to other media commonly used. Nevertheless, it was still necessary to add brain heart infusion to maintain growth of the two spider forms of A. bovis, P2R, and A13R. Although, further simplification of the medium (Georg, Robertstad, Brinkman, and Hicklin, 1964) and its commercial form (BBL) have considerably facilitated its preparation and use, these media, in the opinion of this investigator, have fewer growth promoting properties than the Pine and Watson medium.

With the use of casitone in the basic medium, Pine and Howell (1956) were able to obtain maximal growth rates of certain strains under aerobic conditions. Of the strains tested, however, only 5 were capable of surviving 4 to 6 continuous aerobic transfers in liquid shaking cultures. Under these conditions, carbon dioxide was still required for growth. Under aerobic conditions, the growth of three strains (strains 279, 286, and 296) doubled. Since strains 279 and 286 were recognized strains of A. naestlundii (Howell et al., 1959), this investigator concluded that all three were species of A. naestlundii and that A. naestlundii, as delineated from A. israelii, not only survived in the presence of oxygen, but actively metabolized oxygen with resultant increased cell

yields. Howell, however, maintained that strain 296 (A. israelii, ATCC 10049) was more like israelii than naeslundii (Personal communication). That Howell was correct is discussed later.

Buchanan and Pine (1963) investigated the factors influencing the fermentation and growth of the atypical strain of Actinomyces "naeslundii" (strain 296, ATCC 10049). Casitone was required for aerobic growth. The casitone factor was not identified, but the factor was shown not to be formate, acetate, pyruvate, fumarate, or any of the factors present in the synthetic medium; it could be replaced by using a large inoculum. It was not present in yeast extract, beef extract, blood serum, skim milk neopeptone, and other biological products. Anaerobic growth was unpredictable at pH 7.5, but growth was invariably depressed or failed at this pH under aerobic conditions in the presence of casitone and CO₂. In air, glucose was stoichiometrically oxidized to acetate and carbon dioxide. Determinations of fermentation balances under a variety of conditions and substrates showed the organism was homolactic in the sense of Buyze, Van Den Hamer, and DeHaan (1957), i.e., it contained aldolase. These and similar results relative to L. bifidus, Ar. propionica, Bact. matrochotii, A. bovis, A. viscosus (the hamster organism), and R. dentocariosa (N. salivae) permit a comparison of species on the basis of fermentation balances and give some preliminary insight into metabolic pathways.

Anaerobic and aerobic fermentations of Actinomycetaceae species and related genera.

Fermentation balances of the various species capable of growing under strictly anaerobic conditions are given in Table 3. The fermentations presented may be summarized approximately by the following simplified chemical equations:

A. israelii, A. bovis, A. naeslundii, and A. viscosus

- (1) glucose $\rightarrow$ 2 lactate
- (2) 3.5 glucose + 3 CO₂ $\rightarrow$ 3 formate + 3 acetate + 3 succinate + 1 lactate
- (3) Glucose + 2 malate $\rightarrow$ 2 formate + 2 acetate + 2 succinate

B. bifidum

- (4) 10 glucose + CO₂ $\rightarrow$ 1 formate + 13 acetate + 10 lactate + 1 succinate
- (5) 10 glucose $\rightarrow$ 15 acetate + 10 lactate

Ar. propionica

- (6) 18 glucose $\rightarrow$ 10 CO₂ + 2 formate + 10 acetate + 23 propionate + 1 succinate + 1 lactate

P. pentoaceum, P. arabinosum

- (7) 10 glucose $\rightarrow$ 6 CO₂ + 2 acetate + 14 propionate + 2 succinate

C. acnes

- (8) 10 glucose $\rightarrow$ 5 CO₂ + 4 acetate + 12 propionate + 1 lactate + 2 succinate

The absence of carbon dioxide formation by species of Actinomyces and

Table 3. Anaerobic fermentation balances of Actinomycetaceae and related species.

Organism	growth conditions	Expressed as μM per 100 μM of substrate utilized								%C recd	redox value
		CO ₂ fixed	CO ₂ formed	HF	ETOH	HA	HP	HLA	HSuc		
<i>Actinomyces bovis</i> ¹	glucose + CO ₂ , casitone	35.4	0	35.4	-	33.3	0	125.0	35.4	97.7	1.00
<i>A. israelii</i> ² (10049)	glucose + CO ₂ , casitone	32.7	-	29.3	-	23.7	0	139.5	32.3	98.7	1.06
<i>A. israelii</i> ² (10049)	glucose, limited CO ₂ , casein hydrolysate	-	2.1	23.7	3.7	20.0	0	205.3	n.d.	108.0	3.78
<i>A. israelii</i> ³ (10049)	glucose, casein hydrolysate, No CO ₂	n.d.	-	<5	-	<5	0	192.6	n.d.	96.3	1.00
<i>A. israelii</i> ³ (10049)	glucose, casitone, CO ₂	70.7	0	61.6	-	72.7	0	33.6	75.1	90.7	0.97
<i>A. israelii</i> ³ (10049)	glucose, malate, casein hydrolysate, No CO ₂	n.d.	-	192.7	-	191.4	0	4.3	249.0	97.4	0.76
<i>A. naeslundii</i> ² (strain 279)	glucose, limited CO ₂ , casein hydrolysate	n.d.	2.2	13.2	4.4	6.2	0	195.0	n.d.	98.8	2.00
<i>A. naeslundii</i> ² (strain 279)	glucose, casitone, CO ₂	35.8	0	31.8	n.d.	12.8	0	169.0	18.9	100.7	0.77
<i>A. viscosus</i> ⁸ (hamster organism, T6)	glucose, casitone, CO ₂	3.5	0	3.8	n.d.	1.8	0	166.5	5.0	87.9	0.80
<i>Bifidobacterium</i> ² (<i>Lactobacillus bifidus</i> , strain 308, Timberlain)	glucose, casitone, CO ₂	9.7	0	11.8	n.d.	135.6	0	94.1	7.3	97.3	0.98

<i>Bifidobacterium</i> ⁴ <i>bifidum</i> (308)	glucose, casitone, CO ₂	n.d.	-	4.0	-	141.0	0	99.4	1.2	98.2	n.d.
<i>Arachnia propionica</i> ⁵ (<i>Actinomyces</i>)	glucose, casitone, CO ₂	0	53.5	4.4	-	55.3	114.5	2.5	3.1	104.4	1.00
<i>Ar. propionica</i> ⁵	glucose, casitone, No CO ₂	-	n.d.	0	-	71.5	118.7	2.8	4.9	102.4	-
<i>Propionibacterium</i> ⁶ <i>arabinosum</i>	glucose, N ₂ atmosphere	-	63.6	0	-	17.8	139.1	-	13.2	97.6	1.01
<i>P. pentosaceum</i> ⁶	glucose, under N ₂ atmosphere	-	52.3	0	-	16.6	127.8	-	21.1	95.0	0.98
<i>Corynebacterium</i> ⁷ <i>acnes</i> (strain 11827)	glucose, anaerobic	-	48.0	2.3	-	53.2	115.6	12.2	17.0	1.00 ⁷	1.00 ⁷

1. Pine, Howell and Watson (1960). (Average results taken of 9 strains). HF = formic acid; HA = acetic acid; ETOH = ethanol; HP = propionic acid; HLa = lactic acid; HSuc = succinic acid, n.d. = not determined.

2. Pine and Howell (1956).

3. Buchanan and Pine (1963).

4. Pine (1956; 1960).

5. Buchanan and Pine (1962). Only 16 μ M of glucose/ml were fermented in a med contain 28 μ M/ml.

6. Wood and Werkman (1936). Approximately 150 μ M glucose per ml were fermented.

7. Moore and Cato (1963). Theoretical carbon dioxide and glucose values added by this reviewer to give redox values and % carbon recovered.

8. Howell and Jordon (1963).

Table 4. Glucose "Fermentation balances" of Actinomycetaceae and related species under aerobic or semi-aerobic conditions.

Organism	growth conditions	expressed as μM per 100 μM of glucose utilized						
		CO ₂ formed	HF	HA	HP	HLA	HSuc	%C
<i>Actinomyces israelii</i> ¹ (10049)	shaken, casitone, sealed with rubber stopper	187.6	25.7	169.0	0	7.1	2.4	97.0
<i>A. israelii</i> ¹ (10049)	shaken, casitone, +CO ₂	202.6	0	202.6	n.d.	n.d.	n.d.	101.3
<i>Bacterionema matruchotii</i> ² (strain C-372)	stagnant tubes, casitone	+	1.7	0	60.2	128.8	n.d.	94.8
<i>B. matruchotii</i> ² (strain C-372)	shaken, casitone	+	3.5	30.5	42.3	52.7	n.d.	58.4
"	microaerophilic		2.5	8.4	25.4	78.8	3.3	57.5
<i>B. matruchotii</i> ² (strain CS-552)	microaerophilic deeps, casitone,	+	14.4	31.5	8.5	46.9	1.0	41.3
<i>A. viscosus</i> ³ (hamster organism, T6)	aerobic + CO ₂	n.d.	11.5	11.8	0	100.4	17.1	68.0
<i>Rothia dentocariosa</i> ³ (<i>Nocardia salivae</i>) C442	aerobic + CO ₂	n.d.	0	13.8	0	149.5	1.7	80.4
<i>R. dentocariosa</i> ³ (<i>Nocardia salivae</i>) 24-2A-R	aerobic + CO ₂	n.d.	6.0	10.4	0	153.0	1.0	81.0

<i>Arachnia propionica</i> ⁴	casitone, aerobic shake	169.4	0	157.4	0	0.5	0.5	96.0
<i>Propionibacterium</i> ⁵ <i>arabinosum</i>		200		200				
<i>Corynebacterium acnes</i> ⁶ (strain 11827)	aerobic deep	176.6	(6)	76.6	123.3			

1. Buchanan and Pine (1963). CO₂ not determined in the shaken fermentation; concentration was calculated on the basis of equivalent acetate and CO₂ production.
2. Howell and Pine (1961).
3. Howell and Jordan (1963).
4. Buchanan and Pine (1962). Percent carbon recovered calculated on the basis of the amount of glucose -C¹⁴ incorporated into cell material.
5. Ichikawa (1957). Based on the report by the author of stoichiometric conversion of glucose to acetate plus CO₂.
6. Moore and Cato (1963). "Theoretical" values of glucose and CO₂ calculated by reviewer from original data of author.

B. bifidum distinguishes these organisms from Arachnia, Propionibacterium, and Corynebacterium. Carbon dioxide-C¹⁴ studies (Pine 1956) showed at most a 15 percent dilution of the initial carbon dioxide when glucose was fermented anaerobically by either A. israelii or B. bifidum. On the other hand, both organisms incorporated the carbon dioxide¹⁴ into the carboxyl carbons of succinate (Table 5) Pine, 1969; the data are consistent with the fixation by the "Wood Werkman" reaction. Although this reaction is known to occur in the above species, only species of Actinomyces show a major net fixation of added carbon dioxide with a high production of succinic acid when glucose is fermented.

The formation of succinate by B. bifidum is only a minor reaction, and the overall stoichiometry of the glucose fermentation fits either equation 5 or glucose $\rightarrow$ lactic acid + 1.4l acetic acid + unknown products (Pine, 1956). This fermentation is characteristic and distinguishes this species from all others. Pine and Georg (unpublished) were unable to distinguish between B. bifidum and A. eriksonii on the basis of volatile acid formation, and these organisms are fermentatively similar. The reactions 6, 7, and 8 depicting the formation of propionate from glucose are sufficiently alike that they do not distinguish between the genera which form propionate. But major metabolic differences between Arachnia and Propionibacterium are indicated. Arachnia ferments only 16 micromoles of glucose per ml, whereas members of the genus Propionibacterium may ferment as much as 150 micromoles of glucose per ml. Furthermore, the genus Propionibacterium characteristically readily ferments glycerol and lactic acid with carbon dioxide fixation and the production of propionate; Arachnia species do not ferment lactic acid and attack glycerol extremely slowly, if at all. Although propionate formation would appear to be characteristic of only the species Arachnia and Propionibacterium, Li and Georg (1968) have reported that trace amounts of propionic acid are formed by strains of A. israelii but not by A. naeslundii.

Results of glucose fermentations by the various genera under "aerobic" conditions are presented in Table 4. The condition of aerobiosis varied according to the investigator and the ability of the organism to grow in a strictly aerobic atmosphere in the presence or absence of added carbon dioxide. Under strictly aerobic conditions in the presence of added carbon dioxide A. israelii, Ar. propionica, and P. arabinosum fermented glucose according to the reactions: (9) $\text{glucose} + \text{O}_2 \rightarrow 2 \text{ acetate} + 2\text{H}_2\text{O}$. Strains of B. bifidum tested under identical conditions did not grow. Under conditions described as micro-aerophilic but which were essentially anaerobic, A. viscosus fermented glucose with a net uptake of carbon dioxide and the formation of formate, acetate, lactate, and succinate strikingly similar to that reported for the other species of Actinomyces (Howell and Jordan, 1963) (Table 3). Under more aerobic conditions the carbon recoveries were lower, but greater amounts of formate and acetate accumulated with a concomitant decrease in lactic acid production (Table 4). Rothia dentocariosa (N. salvae) under identical aerobic conditions essentially formed only lactic acid. Comparison of the changes in fermentation products observed under anaerobic and aerobic conditions and a comparison of the relative carbon recoveries suggested that each of the organisms which grew aerobically had an energy producing aerobic metabolism. This is particularly true of a strain of Bact. matruchotii tested (Howell

and Pine, 1961). When the carbon recovery was approximately 95 percent in a Bact. matruchotii fermentation, propionate and lactate were essentially the only products; under shaken conditions the carbon recoveries were approximately 60 percent, and greater amounts of acetate accumulated with a decrease in propionate and lactate. On the basis of its propionic acid formation, the metabolism of Bact. matruchotii is closely related to that of Arachnia and Propionibacterium. All other organisms are related by reactions 1, 2, and 4, whereas B. bifidum is unique by reaction 5.

Metabolic pathways.

The metabolic pathways pertaining to the formation of the described fermentations have in several instances been described in the organisms with which we are concerned. Allen et al. (1964) have described extensively the purification and properties of the enzymes involved in the propionic acid fermentation of P. shermanii. Scardovi and Trovattelli (1965), DeVries, Gerbrandy, and Stouthamer (1967) have described the pathways of glucose fermentation in B. bifidum, and Buchanan (1962) and Buchanan and Pine (1967) have reported on the path of glucose breakdown in Actinomyces israelii and to a limited extent in Arachnia propionica. In addition, extensive reviews by Gunsalus and Shuster (1961) and Wood (1961) describe the metabolic pathways pertinent to each of the organisms. Only the most salient points will be covered here.

Because of the great similarity of the fermentation balances of C. acnes, P. arabinosum, Ar. propionica, and Bact. matruchotii, the mechanism of glucose fermentation in these species is presumably the same, and propionate is formed essentially as described by Allen et al. (1964). In the Propionibacterium the Embden-Meyerhof pathways most probably represent the major pathway of glucose breakdown (Wood, 1961). Pyruvate is converted to oxalacetate via transcarboxylase or phosphoenolpyruvate is converted to oxalacetate by a "Wood-Werkman" reaction. Oxalacetate is then converted to malate, malate to fumarate, and fumarate to succinate. Propionyl-CoA is formed from succinyl-CoA via methyl-malonyl-CoA and then transcarboxylase. The steps through the formation of succinate have been demonstrated enzymatically in A. israelii; absence of the propionate cycle in this organism (and presumably in other species of Actinomyces) is most probably due to the absence of one or all of the enzymes - methyl-malonyl isomerase, methyl-malonyl racemase, or transcarboxylase. However, trace formation of propionate by A. israelii (Li and Georg, 1968) suggests the limited functioning of these enzymes.

Kuhn and Tiedemann (1953) fermented glucose-1- C^{14} with B. bifidum and obtained lactic acid and acetic acids having the same specific activity. $C^{14}O_2$ was not incorporated into lactic or acetic acid. In agreement with Kuhn and Tiedemann, Pine (1956) found no incorporation of $C^{14}O_2$ into these products but found that succinate became highly labeled only in carboxyl carbons (Pine, 1960). Scardovi and Trovattelli (1965) compared the enzymatic composition of Corynebacterium, Actinomyces, Ramibacterium, Arthrobacter, and certain Lactobacillus strains of Bifidobacterium. Of a large number of cell free extracts tested, only the strains of Bifidobacterium were found to have phosphoketolase activity. The authors emphasized, however, that the significance of

this test was obscured by phosphatase activity from some strains which competed with the phosphoketolase. These difficulties were particularly evident with enzymes of A. israelii and A. bovis. Some strains of Actinomyces did not present this difficulty, however, and they concluded that the phosphofructoketolase enzyme was not present within the Actinomyces. Similarly, attempts to demonstrate the presence of phosphofructokinase in four bifid strains failed. However, tests for aldolase were positive in the four bifid strains. When the strains of Bifidobacterium were cultured with glucose-1-C¹⁴, essentially all the radioactivity was found in the methyl carbons of acetic acid only. Their data clearly supported the characteristic functioning of a Schramm, Klybas, and Racker (1958) phosphofructoketolase shunt (Gunsalas and Shuster 1961).

In a thorough enzymatic analyses of the glucose degrading systems of a fermenting strain of B. bifidum, DeVries, Gerbrandy, and Stout-hamer (1967) found the same pattern of glucose 1-C¹⁴ fermentation as reported by Scardovi and Trovatelli (1965). A new dimension pertinent to the glucose breakdown was implicated, however, when it was found that glucose-6-phosphate dehydrogenase was absent and that the aldolase activity was only 0.3 percent that of L. casei. It was on the basis of the presence or absence of aldolase that Buyze, Van Den Hamer and DeHaan (1957) distinguished between the homolactic and heterolactic acid fermentation. Subsequently DeVries and Stouthamer (1967) examined the cell-free extracts of 17 strains of B. bifidum for the presence of the three basic enzymes -- aldolase (indicating the presence of the Embden-Meyerhof scheme), glucose-6-phosphate dehydrogenase (indicating the presence of the hexosemonophosphate shunt), and phosphofructoketolase (indicating the phosphofructoketolase shunt of Schramm, Klybas and Racker, 1958). All strains lacked aldolase and glucose-6-phosphate dehydrogenase, and all contained phosphofructoketolase. On the basis of these results these workers concluded that the classification of the bifidobacteria in the genus Lactobacillus was not justified.

Buchanan (1962) and Buchanan and Pine (1967) investigated the path of glucose breakdown in the genus Actinomyces. The organism used was A. israelii (A. naeslundii) strain 296 (ATCC 10049), since it had a strong aerobic metabolism and would represent the characteristics of both A. israelii, A. naeslundii, and Ar. propionica (A. propionicus). In fermentations of glucose-1-C¹⁴ and glucose-6-C¹⁴ the radioactivity found in formate, carboxyl groups of acetate, lactate, and succinate and that found in the residual carbon dioxide was low (Table 5). The carboxyl groups had approximately 4 to 9 percent of the specific activity of the original glucose-1-carbon; thus we calculated that not more than 6 percent of the total glucose fermented was channeled through either the hexosemonophosphate shunt or the Entner-Duodoroff pathway. However, the specific activities of acetate methyl carbon and the lactate methyl carbon derived from glucose-1-C¹⁴ were 40.0 and 37.8 percent, respectively, of the original glucose-1-C¹⁴, whereas these carbon atoms derived from glucose-6-C¹⁴ were 21.4 and 37.8, respectively. In retrospect, the fact that there was the decreased specific activity of the acetate methyl carbon when glucose-6-C¹⁴ was fermented whereas the specific activity of the methyl carbon of lactate was the same with both glucose-1-C¹⁴ and glucose-6-C¹⁴ clearly indicated a partial functioning of the phosphofructoketolase reaction. The data suggests that approximately 20-50 percent of the acetate was formed this way.

Table 5. Tracer experiments with *Actinomyces israelii* and *Lactobacillus bifidus*.

¹⁴ C substrate	<i>Actinomyces israelii</i> ATCC 10049								<i>Lactobacillus bifidus</i> 308		
	# glucose 1- ¹⁴ C	# glucose 6- ¹⁴ C	# glucose 1- ¹⁴ C	# glucose 6- ¹⁴ C	anaerobic ¹⁴ C ₁₄ O ₂	anaerobic ¹⁴ C ₁₄ O ₂ ‡	aerobic ¹⁴ C ₁₄ O ₂	aerobic ¹⁴ C ₁₄ O ₂ ‡	¹⁴ C ₁₄ O ₂	anaerobic ¹⁴ C ₁₄ O ₂	anaerobic ¹⁴ C ₁₄ O ₂ ‡
	µM/100 µM glucose used										
CO ₂ final	-	-	rsa*	rsa	-	rsa	-	rsa	-		rsa
			5.0	0.2		94.2		49.5			75.6
CO ₂ fixed	52.5	54.9	-	-	56.2	-	-	-	2.5	-	-
acetate	47.1	49.7	45.2	33.6	30.0	0	42.2	0	141.0	0	0
CH ₃	-	-	40.0	21.4	-	-	-	-	-	-	-
COOH	-	-	7.0	9.3	-	-	-	-	-	-	-
formate	48.2	52.9	5.8	0.8	36.6	9.7	47.6	3.6	4.0	0	0
lactate	21.4	23.6	52.7	48.0	125.6	n.d.	95.0	2.0	99.4	0	0
CH ₃	-	-	37.8	37.8	-	-	-	0	-	-	-
CHOH	-	-	6.6	3.8	-	-	-	0	-	-	-
COOH	-	-	4.0	6.5	-	-	-	2.0	-	-	-
succinate	54.9	54.5	53.1	47.5	32.0	100.6 ‡	22.4 ‡	21.3	1.2		26.5
COOH	-	-	2.0	0.4	-	+	-	+	-		+
CH ₂	-	-	23.6	23.5	-	0	-	0	-	-	0
redox	0.98	0.98	-	-	0.56	-	-	-	1.0	-	-
% carbon recvd.	77.9	76.7	-	-	91.6	-	84.2	-	97.6	-	-

* rsa = relative specific activity

= data of Buchanan and Pine (1967)

‡ = data of Pine (1957, 1960)

Enzyme analyses of cell-free extracts established the presence of the major enzymes necessary for the functioning of the glycolytic cycle, hexosemonophosphate oxidative shunt, and the relative pentose cycle (Wood, 1961). However, the rather low activity of glucose-6-phosphate dehydrogenase, the low radioactivity of carbon dioxide from glucose-1- C^{14} , and the very low reduction of specific activity of carbon dioxide in carbon dioxide fixation experiments (Table 5) suggest that the oxidative cycle is limited, anaerobically, to synthetic reactions. All of the citric acid cycle enzymes were demonstrated except alpha-ketoglutaric acid decarboxylase (dehydrogenase) and condensing enzyme (Buchanan, 1962), or specific tracer evidence was presented that they functioned in the growth of the organism (Buchanan and Pine, 1965). Limited enzyme analyses of cell-free extracts from Ar. propionica showed the presence of aldolase, 6-phosphogluconic dehydrogenase, and isocitric dehydrogenase in significant concentrations.

These results indicate that within the groups of organisms just examined glucose degradative systems for the purposes of energy or synthesis are most probably the most complex in the Propionibacterium to which Arachnia is similar. The loss of one enzyme (alpha-ketoglutaric acid dehydrogenase) suffices to eliminate an oxidative tricarboxylic cycle, development of the electron transfer systems notwithstanding. The loss of a single enzyme of three (methyl-malonyl isomerase, methyl-malonyl racemase, or transcarboxylase) would suffice to eliminate the production of propionic acid from A. israelii (or A. naeslundii). Elimination of aldolase combined with the low activity of glucose-6-phosphate dehydrogenase could theoretically shift the metabolism of A. israelii to one similar to that of Bifidobacterium, i.e. to the hexosemonophosphate shunt mediated through phosphofructokinolase.

Cell yields, carbon dioxide, and growth in oxygen. The importance of relating cell yields to the potential catabolic reactions has been reviewed extensively by Gunsalus and Shuster (1961). The Y values (gr dry wt yield of cells per mole of glucose) and the Y_{ATP} values (gr dry wt yield of cells per mole of ATP), as defined by Bauchop and Elsdon (1960), describe whether or not a given metabolic system is actually functional during growth. Cell suspensions of strict anaerobes may well use oxygen to oxidize pyruvate to acetate and carbon dioxide, but such a system does not function successfully in vivo for the release of usable energy.

A universal Y value of 22 and a Y_{ATP} value of 10.5 are generally accepted (Senez, 1962), although these values are subject to criticism (Moustafa and Collins, 1968). Y values or theoretically calculated Y_{ATP} values which are found experimentally to be higher than the accepted values make it suspect that the organism studied has an unknown metabolic process for obtaining additional ATP. DeVries and Stouthamer (1967) have reported the Y value for one strain of Bifidobacterium as 37.4; this high value was explained by the functioning of the phosphofructokinolase system in which two moles of fructose form 5 moles of ATP. Similarly, values of 37.5 for P. pentosaceum have been described by Bauchop and Elsdon (1960). By the reactions known it was postulated that the formation of 1 mole of propionate was associated with the formation of one mole of ATP. Later, Allen et al. (1964) described the reactions of the propionic acid fermentation. The two additional moles of ATP were postulated as being formed by an electron

transport-coupled phosphorylation through the reduction of flavoprotein by reduced coenzyme 1 and H^+ ion. Similarly, Buchanan and Pine (1967) demonstrated that with substrate concentrations of carbon dioxide the Y values obtained with *A. israelii* ranged from 41 to 51. The theoretical Y_{ATP} values of these fermentations were 10.1 to 21.8, depending upon the stoichiometry of the reaction due to conditions of growth and the presence or absence of oxygen and carbon dioxide. The high Y_{ATP} values obtained anaerobically (14.7-18.2) could be explained in part by the functioning of the phosphofructokinolase system described by Gunsalus and Shuster (1961). The data also suggests that the high Y_{ATP} values are related to carbon dioxide fixation and formation of succinate. Thus the high Y_{ATP} values observed for *Actinomyces* would appear to be related to metabolic processes common to *Bifidobacterium* and to *Propionibacterium*.

Buchanan and Pine (1965) have demonstrated that carbon dioxide is required as a growth factor for *A. israelii* for the synthesis of aspartic acid for which the organisms have no permease. As indicated by the fermentation balances, carbon dioxide also functions in the synthesis of succinate; additional energy for growth is released by conversion of pyruvate to acetyl phosphate and formate (Buchanan and Pine, 1963). Finally, carbon dioxide is required by some strains in greater amount to initiate aerobic growth even though they actively form carbon dioxide under these conditions. The relationships, therefore, between carbon dioxide, oxygen, and cell yields in this group of organisms is complex; these relationships, so far as they are known, are summarized in Table 6. The presence or absence of catalase is included in Table 6 because the findings serve as a reference to the mechanism of oxygen utilization or electron transport. The conditions under which the stock cultures may be maintained are also given.

The data in Table 6 emphasize several points. The first five organisms from left to right show a net fixation of carbon dioxide and a progressive increase in aerobic metabolism. Of these species, only *A. naeslundii* does not require carbon dioxide for aerobic growth. Regardless of the cell yields and aerobic metabolism, practice has shown that those organisms which are catalase negative are best maintained under anaerobic conditions. Four species, *A. neaslundii*, *A. viscosus*, *R. dentocariosa*, and *B. matruchotii*, show a doubling of cell yields aerobically as does *A. israelii* strain 10049, provided the casitone growth factor is present.

Catalase and haemoproteins.

The demonstration of catalase is important in relation to an aerobic metabolism; but no assumption can be made that organisms which synthesize catalase can also synthesize cytochromes or have a functioning cytochrome system. Catalase may be of two types, a pseudocatalase which is insensitive to azide or cyanide and a true porphyrin catalase which is sensitive to these reagents (Whittenbury 1964). Deibel and Evans (1960) reported on the benzidine test for iron-porphyrin compounds; using this test, they found that *L. plantarum*, *Pediococcus cerevisiae*, and *Microbacterium thermospactum* were catalase + but benzidine -; yet *Staphylococcus aureus*, *Shigella dysenteriae* and *Pseudomonas* were catalase - but benzidine +. Whittenbury (1964) found that organisms containing the pseudocatalase gave a negative benzidine test

Table 6. Relationships between anaerobiosis, carbon dioxide, oxygen and growth in species of family Actinomycetaceae.

Conditions of growth	<u>Bifidobacterium</u>	<u>Actinomyces</u> <u>eriksonii</u>	<u>A. bovis</u>	<u>A. naeslundii</u>	<u>A. israelii</u>	<u>A. viscosus</u>	<u>Rothia</u> <u>dentocariosa</u>	<u>Arachnia</u> <u>propionica</u>	<u>Bacterionema</u> <u>matruhotii</u>
Net fixation of CO ₂	+	+	+	+	+	+	+	+	+
CO ₂ required for anaerobic growth	+	?	+	+	+	+	no growth	-	-
growth anaerobically increased by CO ₂	-	-	+	+	+	+	no growth	-	?
grows aerobically	-	-	+	+	+	+	+	+	+
aerobic growth requires CO ₂	na	na	+	-	+	+	-	-	-
growth aerobically (in CO ₂) is greater by 2x or more than that anaerobically	na	na	-	+	+	+	+	-	+
growth aerobically changes fermentation signifying complete or partial oxidation of substrate	na	na	na	+	+	+	+	+	+
CO ₂ formed aerobically	na	na	na	+	+	+	+	+	+
Catalase	-	-	-	-	-	+	+	-	+
Stock cultures maintained	anaer	anaer	anaer	anaer	anaer	aerob	aerob	anaer	aerob

Data of Pine and Howell (1956); Pine, Howell and Watson (1960); Buchanan and Pine (1962); Buchanan and Pine (1963); Howell et al. (1959); Howell and Jordan (1963); Howell and Pine (1961). na = not applicable.

when grown in the absence of the iron porphyrin compounds but certain strains showed the presence of true catalase and gave a positive benzidine test when cultured on heated blood media. He presented evidence that these later strains could synthesize an apoenzyme which combined with the preformed heme to form true catalase. Johnston and Delwich (1965) showed that L. plantarum contained both types of catalase if grown on blood media; hematin could not be substituted for blood to activate true catalase activity. Johnston and Delwich (1965) isolated the cyanide-resistant catalase of L. plantarum and found it was negative for both heme and flavin. Other workers have reported that aerobic conditions stimulate the production of catalase activity (Jones, Deibel, Niven, 1964) (Clayton, 1969) but that combination of preformed hemin with the apoenzyme to form catalase occurs in Hemophilus species (Biberstein and Gills, 1961) and in Micrococcus pyrogenes (Jensen, 1957). The factors relating to catalase production have been reviewed, for it is the apparent absence of this enzyme in all the Arachnia strains tested which serves to separate this genus from the species of Propionibacterium. Attempts to stimulate the production of catalase by aerobic growth in the presence and absence of added heme or complex media were unsuccessful; anaerobic cultures of Propionibacterium species which were catalase negative upon receipt became strongly catalase positive after several transfers on the Pine and Watson medium, whereas Ar. propionica remained catalase negative. But a consideration of the factors involved in the loss of catalase activity makes the absence of this enzyme in Ar. propionica a minor factor for consideration of phylogenetic relationships. Further studies, such as the testing of heated blood for the production of catalase in Arachnia, could well eliminate even this difference between the two genera. Studies are also needed to determine the nature of the catalase present in the R. dentocariosa and A. viscosus species, for of the Actinomyces species, only A. viscosus contains catalase. Thus, from a phylogenetic standpoint, the presence of the porphyrin enzyme would constitute a major advance in synthetic steps and physiology over that of other Actinomyces species and would warrant a return of the species to a separate generic designation.

Dolin (1969) has suggested that the lactic acid bacteria may represent different stages of physiological development; Streptococcus faecalis has no demonstrable cytochrome yet contains an active cytochrome reductase, whereas pediococci appear to have a cytochrome oxidase activity, since they oxidize phenylenediamine. However, Whittenbury (1964) points out that the ability of some lactics to form haemoproteins only when provided with a source of iron porphyrin suggests that, instead of evolving toward an aerobic physiology, they actually retrogressed from a facultative aerobic haemoprotein-forming organism.

A consideration of the steps involved in the synthesis of iron porphyrin compounds through the intermediates to a functional cytochrome electron transfer system can only impress one as to its complexity and relative specificity of function. But this system can be bypassed in a single step by the function of catalase (or peroxidase), if not for energetic reasons, at least for synthetic ones (Dolin, 1961). From the distance at which we must view the object, it is difficult to determine in which direction the evolutionary steps have moved. Of the members of the proposed family Actinomycetaceae, those which are catalase positive

and aerobic must be recognized as having the most complex anabolic and catabolic systems.

Cell wall composition.

Table 7, which is taken in part from a report to the committee by John M. Slack, shows the qualitative cell wall compositions of the 10 strains considered for the family Actinomycetaceae. The information is a compilation of individual studies by C. S. Cummins (1962) and other members of the committee and data from publications of Cummins and Harris (1956, 1958); Cummins, Glendenning, and Harris (1957); Becker, Lechevalier (1965); Davis and Freer (1960); Pine and Boone (1967), and DeWeese, Gerencser, and Slack (1968). Additional related species are included in the table for comparative purposes and for elucidation of potential relationships.

Of the 10 strains, eight contain lysine as the dibasic amino acid of the murein; Ar. propionica and Bact. matruchotii contain LL-diaminopimelic acid (DAP) and meso-DAP, respectively. Ornithine may be the only dibasic amino acid present in B. bifidum; lysine plus ornithine has also been reported (Cummins, Glendenning, and Harris, 1957). Veerkamp, Lambert, and Saito (1965) have reported the cell wall composition of L. bifidus variety pennsylvanicus; the major amino acids found were aspartic acid, alanine, glutamic acid, serine, and ornithine. Rhamnose, glucose, and galactose constituted the major sugars of the cell wall polysaccharide. Only two species, A. bovis and A. naeslundii, do not contain galactose. Instead, they contain glucose and methyl pentoses. These two species may be distinguished from one another on paper chromatograms by the presence of aspartic acid in A. bovis and the low concentration of aspartic acid in A. naeslundii (Pine and Boone, 1967) and by the presence of ornithine in A. naeslundii (DeWeese, Gerencser, and Slack, 1968). However, the "spider" mutants (P2R, 13R) of A. bovis are without aspartic acid. Furthermore, the quantitative findings of DeWeese, Gerencser, and Slack show aspartic acid is a major constituent of both A. israelii and A. naeslundii. A. israelii strains have alanine, lysine + ornithine, glutamic acid in their murein; galactose is the sugar of the polysaccharide. Because of its high rate of growth, its high cell yields in the presence of oxygen, and its metabolism, as stated earlier A. israelii ATCC 10049 was described as an atypical A. naeslundii. However, analysis of its cell wall showed high concentrations of galactose with minor amounts of rhamnose and mannose (Pine and Boone, 1967). Aspartic acid was consistently present in molar concentrations of 1 relative to 4 of alanine (Pine and Boone, 1968) (DeWeese, Gerencser, and Slack, 1968). On the basis of its high galactose content and because it reacted homologously with anti-A. israelii serum (Lambert, Brown, and Georg, 1967), strain ATCC 10049 was re-classified as A. israelii (Buchanan and Pine, 1967).

In an extremely comprehensive review "Use of Bacteriolytic Enzymes in Determination of Wall Structure and their Role in Cell metabolism", Ghuyssen (1968) describes the known and postulated steps necessary for the coordinated synthesis of the murein (peptidoglycan) and the polysaccharide moieties of bacterial cell wall. Thirty to 60 different enzymes are envisioned as necessary to carry out the cross linkage steps required between the muramic acid, the hexosamine, and the amino acids of the peptide bridges; of the murein (mucopeptide),

Table 7. Cell wall composition of Actinomycetaceae and related species.*

	catalase	aspartic	lysine	ornithine	glycine	DAP	galactose	mannose	glucose	rhamnose	fucose	deoxytalose	arabinose
<i>Lactobacillus acidophilus</i>	-	+	+						+				
<i>L. delbrueckii</i>	-	+	+				+		+	+			
<i>L. plantarum</i>	+					meso			+				
<i>Streptococcus pyogenes</i>	-		+							+			
<i>Bifidobacterium bifidum</i>	-			+			+		+				
<i>Actinomyces eriksonii</i>	-	+	+				+				•		
<i>A. israelii</i> strain ATCC 12102	-		+	+			+						
<i>A. israelii</i> (296) ATCC 10049	-	±	+	?			+	+		+			
<i>A. bovis</i> ATCC 13683	-	+	+					+	+	+	+	+	+
<i>A. bovis</i> P2R; <i>A. bovis</i> 13R	-		+					+	+	+	+	+	+
<i>A. naeslundii</i> (279) ATCC 12104	-	±	+	+				+	+	+	+	+	+
<i>A. odontolyticus</i>	-		+	+			+		+	±	±	±	
<i>A. viscosus</i>	+		+	+			+	+	+				
<i>Rothia dentocariosa</i>	+		+				+						
<i>Arachnia propionica</i>	-				+	LL	+		+				
<i>Bacterionema matruchotii</i>	+					meso	+	+	+				+
<i>Propionibacterium</i>	+				+	LL	+		+				
<i>Corynebacterium acnes</i>	+				+	LL	+		+				
<i>C. diphtheriae</i>	+					meso	+						+
<i>Mycobacterium smegmatis</i>	+					meso	+						+
<i>M. tuberculosis</i>	+					meso	+						+
<i>Nocardia asteroides</i>	+					meso	+		+				+
¹⁰ <i>Micromonospora</i> -type ¹¹	+				+	meso							
¹¹ <i>Streptomyces</i> -type ¹¹	+				+	LL							
<i>Micrococcus aureus</i>	+					+							

* All strains have alanine and glutamic acid. + = component designated is present. Empty squares = component is absent.

and in the cell wall polysaccharide. Thus, of the cycles or integrated systems which we have so far considered, the synthesis of the bacterial cell wall represents one of the, if not the most, complex systems pertinent to establishing phylogenetic relationships. Kandler and his associates (as presented in Ghuysen's review) have classified lactobacteria according to the peptide bridge (linkage groups) found in approximately 50 strains of Lactobacillus, Streptococcus, and Leuconostoc. Four separate groups were designated. The groups were those based on L-amino acid residues only, glycine residues only, glycine and L-amino acid residues, and finally, bridges of single D-isoasparaginyl residues.

The cell wall composition can vary within a given species or strain, depending upon the mechanism of change. Thus, a culture of either smooth or rough A. bovis cells can give rise to a mycelial (spider) mutant whose cell walls are devoid of aspartic acid, but have the same ratio of alanine, glutamic acid and lysine as the mother strain. Somewhat similar morphological changes in growing cultures of A. israelii (ATCC 10049) were not associated with qualitative changes, although certain quantitative changes (increase of lysine and hexosamine) were observed (Pine and Boone, 1967). Similarly, Young (1965) has demonstrated a progressive increase in the alanine content of the cell walls of Bacillus subtilis during a 24 hr period of growth, and variation of alanine content with changes in medium. Brumfitt, Wardlow, and Park (1958) have associated the development of lysosyme resistance in Micrococcus lysodiekcticus with an increased O-acetyl content of the cell wall; the development of variants within the group A- streptococci has been shown to be the result of deletion of N-acetylglucosamine residues from the terminal rhamnose of the group polysaccharide (Krause, 1963).

Although quantitative variations of cell wall composition occur with certain environmental changes, major or minor qualitative changes can occur with mutation. Although morphology may change concomitantly with mutation, this reviewer does not subscribe to the implication that cell wall (composition) dictates normal morphology (Martin, 1966); instead the site of cell wall synthesis is considered to govern, primarily, the morphological aspects of the organism. Thus, the morphological changes observed in the smooth and spider mutants of A. bovis are more likely due to enzymatic changes or site of cell wall synthesis than to a loss of aspartic acid as a building block of the cell wall. It is difficult to envision how any major differences in the murein tetrapeptide (alanine, L-lysine or DAP, D-glutamic acid, and D-alanine) could occur within closely related organisms. The tetrapeptide is considered as a unit and is peculiarly resistant to enzymatic attack (Strominger and Ghuysen, 1967). Substitution of lysine by DAP would not appear feasible without other major sequential changes. Thus, organisms having DAP would appear to be phylogenetically distant from those having lysine as the dibasic amino acid for the tetrapeptide. It is easier to envision how minor changes of the peptide bridges or minor changes of the cell wall polysaccharide occur with concomitant changes in antigenic specificity or even structure (spheroplast).

Serological studies.

The results of serological studies taken directly from a report to the committee by John M. Slack, are given in Table 8. Pertinent references have been added.

Table 8. Serological relationships of species proposed for the family Actinomycetaceae.

Serotypes	A. bovis 13683 1,2	A. eriksonii 15423 1	A. israelii 12102 1,2	A. naeslundii 12104 1	A. odontoly- ticus 17929 1	A. viscosus 15987 1,2	Ar. propionica 14157 1,2	Bact. matru- chotii 14266 1	Bif. bifidum 17930 1	R. dentocariosa 17931 1
(1) <u>F. A. antisera</u>										
A. bovis	4+									
A. eriksonii		4+								
A. israelii U.			4+	2+						
" S.			4+		4+					
A. naeslundii U.			2+	4+						
" S.				4+						
A. odontolyticus					4+					
A. viscosus						4+				
Ar. propionica							4+			
B. matrucho- tii								4+		
B. bifidum									4+	
R. dentocariosa										4+

(1) F. A. = fluorescent antibody.

Slack, Moore, and Gerencser (1966); Slack and Gerencser (1966); Lambert, Brown and Georg (1967); Blank and Georg (1968); Slack, Lanfried and Gerencser (1969); Brock and Georg (1969).

Discussion and conclusions based upon the above considerations.

The family Actinomycetaceae has been considered a morphological group and there is no question that from the practical standpoint "microscopic" morphology is the first step towards identification. On this basis, the present group of 10 species was assimilated and proposed within the last 12 years. The organisms of this group range from anaerobic bifid or diptheroid cell types which do not form mycelium to those which are strictly aerobic and possess, at least for a short period of growth, complicated mycelium and exhibit unique steps in cell multiplication. Their cell wall compositions are diverse. Although morphology originally served to bring these species under scrutiny, this phylogenetic property alone cannot serve to unify them.

Based on the new information, a revised scheme showing certain phylogenetic relationships is presented for these species within the family Actinomycetaceae or related families (Figure 1). The basis for the present taxonomic and phylogenetic considerations has been expressed previously (Pine and Georg, 1965). The ten species can be divided into three fermentation groups on the basis of the products formed: (a) heterolactic acid fermentation, (b) homolactic acid fermentation, and (c) propionic acid fermentation. Eight species comprise groups (a) and (b) and these homolactic and heterolactic acid fermenters are apparently closely related by both the phosphofructotetolase relationships and carbon dioxide relationships (i.e., carbon dioxide is not formed but is fixed in the carboxyl carbons of succinic acid). In addition, the 8 species comprising this group all have lysine or lysine plus ornithine as the dibasic amino acids of their cell wall murein. Apparent relationships among these species, as determined by cell wall compositions and physiology, are shown in Figure 1.

The two species comprising the propionic acid fermentation group shown in Figure 1 are closely related to the Propionibacterium by the presence of DAP, glucose, and galactose in their cell walls. B. matruchotii is closely related to C. diphtheriae and M. tuberculosis by having the meso-isomer of DAP. On this basis, B. matruchotii is conveniently placed between C. diphtheriae and M. tuberculosis; it is related to C. diphtheriae by its propionic acid fermentation but related to M. tuberculosis by virtue of its "acid fast" staining and "mycelial" morphology. Schmidt and Richardson (1962) have shown that B. matruchotii cross reacts strongly with antisera against Mycobacterium, Nocardia, C. xerosis, C. diphtheriae, and Actinomyces in the complement fixation test. Ar. propionica, like Propionibacterium, has LL-DAP. The fact that Ar. propionica is closely related to the genus A. israelii in its morphology, absence of catalase, pathogenicity, and ability to produce "typical actinomycosis with granules" has been presented as reason for its retention within the family (Pine and Georg, 1969). Since the cell wall composition is the factor most pertinent to phylogeny and the absence of catalase is a minor factor, Ar. propionica is more appropriately placed in the family Propionibacteriaceae. Unfortunately, the final position of B. matruchotii is less certain; but its position within the family Actinomycetaceae would appear less suitable than a position within the family Mycobacteriaceae or Corynebacteriaceae where the close relationships are apparent. Here the evaluation of a propionic fermentation suggests the Corynebacteriaceae as more suitable for B. matruchotii.

Figure 1. Phylogenetic relationships among members of the Actinomycetales.

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