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Morphology of the Bifid Bacteria (Organisms Previously Incorrectly Designated *Lactobacillus bifidus*) and Some Related Genera

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SUMMARY: Four morphologically different types of the bifid bacteria can be distinguished, and a classification of these bacteria on morphological basis agrees rather well with earlier groupings based on biochemical and serological characteristics. Morphological features may thus have a taxonomic value as regards the bifid bacteria. A comparison of the morphological characteristics of the bifid bacteria with those of representatives of the genera *Corynebacterium*, *Actinomyces*, *Propionibacterium*, *Butyribacterium* and *Lactobacillus* revealed that three out of four morphological subtypes showed closest resemblance to the genus *Butyribacterium*, whereas the fourth subtype (the one referred to in the literature as *Lactobacillus bifidus* var. *pennsylvanicus*) may be related to *Actinomyces*. Thus, the designation *Lactobacillus bifidus* for these bifid bacteria seems clearly wrong.

The taxonomic relationships of the Gram-positive, anaerobic, asporogenous bacteria of intestinal origin, which are often referred to as the bifid bacteria, seem not to have been clearly defined. Most current schemes of bacterial taxonomy include these organisms in the genus *Lactobacillus* (viz. *L. bifidus*), but strong evidence has been presented to invalidate such classifications (e.g. Negroni & Fischer, 1944; Olsen, 1949; Frank & Skinner, 1954; Hayward, Hale & Bisset, 1955). On the other hand, different proposals for reclassification have been presented, and it has been difficult to decide whether the bifid bacteria are to be transferred to the genus *Corynebacterium*, or to the genus *Actinomyces*, or whether they constitute a distinct group of their own (cf. Prévot, 1938). The problem also serves topical interest, because a number of distinct subtypes of the bifid organisms have been recently described (György, Norris & Rose, 1954; Petuely, 1957; Dehnert, 1957; Gyllenberg & Carlberg, 1958*a, b*). Among these the characteristic intestinal inhabitant of breast-fed infants (cf. Petuely, 1957; Gyllenberg & Carlberg, 1958*a*), and the type requiring specific amino sugars as essential nutrients (named *L. bifidus* var. *pennsylvanicus* by György *et al.*, 1954), deserve particular attention.

In spite of the polymorphic nature of the bifid bacteria, morphological characteristics have always been given very great weight in the discussions concerning their taxonomic relationships. In the present study, too, attention was paid to these characteristics, employing the technique of continuous examination of the cultures at different stages of growth, which method has proved successful in a study on *Arthrobacter* (Sundman, 1958).

As already pointed out, the similarity between the bifid bacteria and

several other groups of Gram-positive, micro-aerophilic or anaerobic bacteria (particularly corynebacteria and actinomycetes) has been demonstrated by different authors, and, therefore, representatives of these groups, as well as of the genera *Butyrubacterium*, *Propionibacterium* and *Lactobacillus*, were included in the study to permit comparison. As regards the corynebacteria, it was assumed that *Corynebacterium acnes* as an obligate to facultatively anaerobic organism is more closely related to the bifid bacteria than are the aerobic corynebacteria. *C. acnes* was selected, therefore, to be the representative of this group.

METHODS

Organisms. Thirteen strains of bifid bacteria were chosen as typical for some hundred isolates from seven faecal samples of breast-, bottle- and spoon-fed infants. The isolates had been obtained, as previously described (Gyllenberg & Carlberg, 1958*a*), by picking all colonies from suitable dilution plates on a highly selective substrate developed by Petuely (1956). This substrate is described below under 'culture media' as the P-agar. After the purity of the isolates had been checked during a few passages as surface streaks on the more complex G-agar, they were kept as stock cultures in tomato agar slabs. The 13 cultures included in the present study are the same as described by Sundman & af Björkstén (1958) and by Gyllenberg & Carlberg (1958*b*): A1, A2, A4, A6, B4, B11, B18, C8, C25, D1, E15, K17, M1. Further, the strain ATCC 11863, received in 1956, was included. The three first-mentioned cultures are lacking in Table 1 of Gyllenberg & Carlberg (1958*b*) where the nutritional demands and the fermentation reactions of the strains are listed, but they are comparable with the strains A6 and E15 in that table. Of the cultures included in the present investigation, the strains D1, K17, and M1 were representative of the characteristic 'breast-fed infants' type', which we refer to as *type A* (cf. Gyllenberg & Carlberg, 1958*a*; Sundman & af Björkstén, 1958); the cultures B4, B11, and 11863, again, were identified as *Lactobacillus bifidus* var. *pennsylvanicus* (György *et al.*, 1954), but are referred to as *type C* in this report. The other cultures (A1, A2, A4, A6, E15, B18, C8, and C25) were nutritionally identical but differed in fermentation characteristics in that the five first-mentioned cultures utilized pentoses but not sorbitol, while the three last-mentioned cultures behaved vice versa (cf. Gyllenberg & Carlberg, 1958*b*; Sundman & af Björkstén, 1958). We prefer, therefore, to distinguish between these cultures (cf. Dehnert, 1957), and to refer to them as *type B₁*, and *type B₂*, respectively. All our isolates of bifid bacteria were strictly anaerobic, no adaptation to aerobic growth was ever noticed.

The following five cultures of the genus *Propionibacterium* were obtained from the Department of Dairy Science, University of Helsinki, through the courtesy of Dr M. Antila: *P. freudenreichii* NCIB 8095, *P. thoenii* NCIB 5966, *P. zeae* NCIB 8100, *P. pentosaceum* NCIB 5961 and *P. intermedium* NCIB 8071.

Butyrubacterium rettgeri was kindly supplied by Dr H. A. Barker, University of California, Berkeley.

Four strains of *Corynebacterium acnes* were investigated, viz. NCTC 737,

received in October 1956, the strains R68 and AD, isolated by Gyllenberg (1958) as specific contaminants in cultures of bifid bacteria, and the strain ATCC 11617, received as *Lactobacillus bifidus*, but later identified as *C. acnes*.

Two cultures of *Actinomyces bovis* of human origin—A1 and A5, representatives of serological groups A and B respectively (Slack *et al.*, 1955)—were kindly put at our disposal by Dr J. M. Slack, West Virginia University, Morgantown, U.S.A. Unfortunately the sign A1 is the same as used in this laboratory for one of the bifid strains. To avoid confusion and to make it possible to refer to earlier descriptions of the strains (Slack *et al.*, 1955; Sundman & af Björkstén, 1958), the nomenclature is not changed.

The following *Lactobacillus* strains were included: *L. helveticus* strains L1 and L4, from the Department of Dairy Science, University of Helsinki; *L. bulgaricus*, isolated in this laboratory from marketed yoghurt; *L. fermenti*, two strains of which (BS21 and BN) were kindly sent to us by Dr Elisabeth Sharpe, National Institute for Research in Dairying, Reading, and the strain V8 which was isolated in this laboratory from infant faeces.

Culture media. The tomato agar used was the same as described by Sundman & af Björkstén (1958). For the propionic acid bacteria this substrate was modified to contain glucose instead of lactose, and referred to as tomato agar with glucose. The G-agar was previously described by Sundman & af Björkstén (1958). The ascorbic acid and the lactose of this substrate were sterilized separately by filtration and added immediately before use to the rest of the medium sterilized in the autoclave. The P-agar was prepared in the same way and contained per 100 ml. of final medium: ascorbic acid, 1 g.; lactose, 3.5 g.; K_2HPO_4 , 0.5 g.; $(NH_4)_2SO_4$, 0.25 g.; cysteine hydrochloride, 0.04 g.; Ca pantothenate, 50 μ g; biotin, 0.5 μ g.; N-acetyl-d-glucosamine, 20 mg.; Tween 80, 0.5 ml.; 'salts B' solution, 0.5 ml.; agar, 2.5 g.; pH 6.6. The actinomycetes were grown on the CYE substrate according to Howell & Pine (1956), omitting the powdered $CaCO_3$ and substituting the 5% (v/v) CO_2 +95% (v/v) N_2 atmosphere with CO_2 only. The lactate agar employed for the propionic acid had the following composition: peptone Difco, 2 g.; sodium lactate, 1 g.; yeast extract Difco, 1 g.; agar, 1.5 g.; distilled water, 100 ml.; pH 7.0.

General procedure. For continuous examination of morphology, agar plates were inoculated by spreading a small volume of a suspension of the stock culture (tomato agar stab when not otherwise stated) over the surface. The excess of the suspension was poured off and the plates incubated, according to the demands of the organism in question, either with free access to the air or in an desiccator which was evacuated to 15–20 mm. Hg and subsequently filled with CO_2 . After various intervals agar blocks were cut out of the plates, and contact prints made for microscopical examination. When, at later growth stages, the compact cell mass gave contact prints too dense for good preparations, ordinary unfixed smears were examined. Main interest was paid to the shape of the living cells as revealed by the phase contrast microscope in water mounts, and to the site and frequency of cross-septa in stained cell-wall preparations. The micrographs were taken on Adox film R14 Pan with a

primary magnification of $\times 830$, using Leitz' microscopical equipment. For phase contrast photography we mostly used 12 sec. exposure; for bright field photography of stained preparations, 5–6 sec.

Staining methods. The long exposure time made it difficult to get sharp pictures of water mounts when the cells were very susceptible to Brownian movement. In those cases the print or smear was mounted in dilute crystal violet, 0.02 % (w/v) water solution. This decreased the movement of the cells, and when examined by aid of the ordinary bright field microscope these crystal violet mounts had much of the advantages of water mounts in phase contrast microscopy, and were decidedly better than ordinary stained smears as to the details of the cell contours and evenness of the preparation.

Several cell-wall staining methods were tried. The ordinary tannic acid–crystal violet method (Robinow, 1946) gave diffuse and unsatisfactory preparations of most strains studied. The stronger crystal violet employed in the method of Webb (1954) gave better results, but the mordanting (1 hour 5 %, w/v, tannic acid) was obviously insufficient for the actinomycetes and some of the bifid bacteria. The phosphomolybdic acid + methyl green method (Hale, 1953), the crystal violet–Congo red method (Chance, 1953) and the direct staining method with Victoria blue (Robinow & Murray, 1953) did not give satisfactory results. After some experiments the following two modifications of the methods mentioned above were found most suitable.

1. Phosphomolybdic acid + crystal violet method. The unfixed print or smear was mordanted in 1 % (w/v) phosphomolybdic acid for 5–10 min., washed in tap water, stained with 0.5 % (w/v) crystal violet for 15 sec., washed in tap water, mounted in water.

2. Tannic acid + crystal violet method. The unfixed print or smear is mordanted in 5 % (w/v) tannic acid for one day, washed in tap water, stained with 0.5 % (w/v) crystal violet for 3 min., washed in tap water, studied unmounted. The latter method has some disadvantages; it is time consuming and the prolonged mordanting tends to give a background on the slide. It was used when method No. 1 failed to give satisfactory preparations.

RESULTS

The bifid bacteria. As previously reported (Sundman & af Björkstén, 1958), the morphology of the bifid bacteria when grown on tomato agar—a substrate which, with minor modifications, has often been used for cultivation of bifid bacteria—may be abnormal in spite of good growth. Protoplast-like bladders and very pronounced branchings appear on this substrate. Since branching seems to be prevented when the cultures are grown under nutritionally satisfactory conditions (Sundman & af Björkstén, 1958), it is our opinion that the unbranched rod form represents the normal form of growth of these bacteria. Pl. 1, fig. 1 and fig. 2 show the branched and unbranched form, respectively, of strain M1 belonging to the subtype A. Petuely & Eichler (1954) came to the same conclusion, and supposed that the rod forms dominate *in vivo*.

It was the scope of the present investigation to compare the morphology of normally growing cultures of the bifid bacteria. The G agar was hence preferred, particularly since it satisfied the nutritional demands of all strains. The only morphologically distinguishable growth cycle noticed on G-agar was a slight tendency to form longer cells at young growth stages (about 15 hr.). The results reported earlier (Gyllenberg, 1955), describing a pronounced variation in morphology with the age of the culture, could not be confirmed with any one of the 14 strains of bifid bacteria studied (cf. Gyllenberg, 1958). It was found most satisfactory to describe the morphology in well developed cultures (24 hr. or more). Although the morphology of the bifid bacteria was more uniform on G-agar than on the poorer tomato agar, it has to be pointed out that even on the former substrate the morphology of our cultures was rather heterogeneous. The following account of the different subtypes is an attempt to find out the specific characteristics of each type rather than to give a description which covers every organism in the cultures studied.

Three isolates were on physiological grounds identified as type A (Gyllenberg & Carlberg, 1958*b*). They were all morphologically similar, though not offering such evident type-characterizing features as the other types described below. The prevailing form of type A in aged cultures was slightly bent, slender rods, 0.2 to 0.4 by 3 to 7 μ , frequently somewhat swollen in one or both ends. A granule, optically more dense than the rest of the plasma, was often discernible in the swollen ends with the aid of the phase contrast microscope (Pl. 1, figs. 2–3). This optically dense granule can be regarded as a characteristic of type A. Branches were uncommon. When occurring, they appeared either as rather long, terminal bifurcations or as lateral, rounded buds (Pl. 1, fig. 4.) The rods were separated (Pl. 1, fig. 5), the cross-walls often asymmetrically located.

All the strains identified as type B₁ formed a morphologically well separated group characterized by thick even rods of 0.7 to 0.9 by 2 to 3 μ with very few, bud-like, bifid branchings. Pl. 1, figs. 6–9 illustrate the appearance of type B₁. The organisms were occurring separately or grouped in pairs, unicellular, occasionally divided by one central cross-wall into two equal parts. The bud-like branchings were either real branches or separated by a septum from the rest of the organism (Pl. 7, figs. 8–9).

Type B₂ was represented by three cultures. These strains also showed common characteristics separating them from the other isolates of bifid bacteria studied. The curved rods were rather thick, 0.5 to 0.7 by 2 to 8 μ . Branchings were very frequent and mostly located as bilateral, adjacent buds on the middle of the organisms. This resulted in a buffer-like or cross-like appearance (Pl. 2, fig. 10). The ends of the rods were mostly swollen and often square cut in connexion with a curvature at the very end of the rod. These horn-like branchings and rods, observed only in the cultures of type B₂, are seen on Pl. 2, figs. 10–11. The rods were mostly unicellular (Pl. 2, fig. 12).

The three cultures of type C (*Lactobacillus bifidus* var. *pennsylvanicus*) formed a morphologically distinct group. The cultures consisted of two kinds

of cells; slender rods and clavate swellings. The rods were about 0.4μ in diameter, varying in length from 3 to 5μ (Pl. 2, fig. 18); the clavate organisms were up to 1.5μ in diameter and often very common. In smears from aged cultures it was easy to find microscope fields where almost every organism was clavate. The swellings were accompanied by a slightly bifid structure of the opposite end of the organism, resulting in a peculiar amphora-like appearance, schematically drawn in Fig. 1 *a* (cf. Pl. 2, fig. 14). The amphora-like organisms are single or grouped in pairs as seen in Fig. 1 *b*, septated as seen in the figure, and consequently composed of 2 to 4 cells (Pl. 2, fig. 15). These forms were typical for the cultures of type C, less frequent in 11863 than in B4 and B11, but not found in the other types of bifid bacteria. The rod form in the type C cultures was septated (Pl. 2, fig. 16). In an earlier report (Sundman & af Björkstén, 1958, Pl. 2, figs. 8–11) four photomicrographs illustrating the morphology of the subtypes of bifid bacteria were published. In order not to increase the illustrations in the present paper, these photomicrographs are not republished, but referred to, as being illustrative of the present topic.

Fig. 1. Schematic presentation of amphora-like organisms found in cultures of type C of the bifid bacteria: (a) amphora-like organism; (b) peculiar grouping and septation.

The above description of the morphology of the different types of bifid bacteria seems to confirm the earlier mainly physiological classification (Dehnert, 1957; Gyllenberg & Carlberg, 1958*b*). The types B₁ and B₂, corresponding to the groups V and III of Dehnert, respectively, were by Gyllenberg & Carlberg considered as one type named B and characterized by requirements for cysteine (cystine), pantethine (a few strains are capable of growth with pantothenic acid) and riboflavine. The morphological features of the strains separated them in the same distinct groups as Dehnert's classification based on fermentative powers. Our type C (*Lactobacillus bifidus* var. *pennsylvanicus*) corresponds to Dehnert's group I, characterized by rough growth. The smooth form of *L. bifidus* var. *pennsylvanicus* (Dehnert's Group II) was never noticed among our isolates. In Table 1 are collected some data about the four different types of bifid bacteria observed in the present study.

Corynebacterium acnes. This species, found to be the specific 'straight rod' contaminant of cultures of the bifid bacteria (Gyllenberg, 1958), is described as small rods, slightly club-shaped when grown anaerobically. Aerobic conditions are said to induce longer cells and rudimentary branchings (Douglas & Gunter, 1946; *Bergey's Manual*, 1957). All the cultures included in this work formed long branched rods at early growth stages (about 15 hr.) when grown on G-agar in CO₂ atmosphere at 37°. The filamentous rods were 0.2 to 0.4μ in

diameter. Later, when the growth had become visible to the naked eye (about 24 hr.), the filaments had broken up into unbranched rods which continually became shorter and after 2–3 days adopted the shape known as typical for anaerobically grown *C. acnes* (Pl. 3, fig. 17). In spite of many attempts with different staining methods we were not able to demonstrate the location of cross-septa in the branched, filamentous growth stages.

Table 1. *Subdivision of the bifid bacteria*

Type (Gyllenberg)	Group (Dehnert)	Basic demands of growth factors*	Characteristic carbohydrate fermentation abilities†	Morphological characteristics on G-agar
A	IV	B, C, Pa	xylose	slender, swollen rods with optically dense granula
B ₁	V	C, P, Ri	xylose, arabinose, melezitose	even, stout rods
B ₂	III	C, P, Ri	melezitose, sorbitol	branchings, horn- like structures
C	I	C, P, Ri, PPB, As‡	—	1. slender rods 2. amphora-like swellings

* B = biotin, C = cysteine, Pa = pantothenic acid, P = pantethine, Ri = riboflavine, PPB = purine and pyrimidine bases, As = specific amino sugars.

† All strains ferment at least glucose, lactose and saccharose (cf. Dehnert 1957).

‡ Required by freshly isolated strains. Some strains also need a specific peptide factor.

The occurrence of a growth cycle in the cultures of *Corynebacterium acnes* separated this species from all cultures of bifid bacteria included. Even the morphological similarity between, on one side, *C. acnes* at the filamentous and early rod stages and, on the other side, the bifid bacteria, was only superficial. The branched filaments in young *C. acnes* cultures were irregular and of a myceloid appearance not found in the bifid bacteria (Pl. 3, fig. 18). The branches of *C. acnes* rods developed from pointed, lateral buds (Pl. 3, fig. 19), whereas the lateral buds of the bifid bacteria (type A) were rounded (cf. Pl. 1, fig. 4). The bifurcations, seen in all cultures of the bifid bacteria, were never found in the *C. acnes* isolates. Further, *C. acnes* exhibited a constant morphology on G-agar and on tomato agar, whereas the bifid bacteria showed great variability.

Actinomyces bovis. After one subculture in CYE substrate both strains of *A. bovis* were found to contain, beside the filamentous, branched myceloids, also small numbers of cocci. These were supposed to be commensals as described by Erikson & Porteous (1955). Since, nevertheless, the growth on repeated subculture in the semi-solid medium was coherent and filamentous without any turbidity of the substrate, viz. typical for rough *Actinomyces* species, the commensals were assumed not to cause any major disturbance to the host. According to Erikson & Porteous (1955) the fragmentation of *A. bovis* (by these authors named *A. israeli*) may be induced by commensals harboured in small numbers in the *Actinomyces* culture. The influence is said mainly to

cause a variation $R \rightarrow S$ which in the present work was not the case, since the cultures remained rough and the club-shape, typical for rough actinomycetes, prevailed in aged cultures. Morphologically the two *A. bovis* strains were similar. In young cultures the irregularly branched long filaments prevailed (Pl. 3, fig. 20). With ageing of the culture the filaments divided, and shorter, often club-like fragments developed (Pl. 3, figs. 21–22). The cross-walls developed irregularly, as seen on Pl. 3, fig. 23, where the cells are of various lengths. The club-shaped fragments, seen on Pl. 3, fig. 22, show resemblance to cell forms seen in type C of the bifid bacteria (cf. Pl. 2, fig. 15).

Propionibacterium. The descriptions of the morphology of the propionic acid bacteria in the literature (cf. *Bergey's Manual*, 1957) mostly refer to the work of van Niel (1928). Anaerobic growth is described as circular to short cells, singly, in pairs or in short chains, aerobic growth as irregular club-shaped and branched rods. The 5 strains: *P. freudenreichii*, *P. thoenii*, *P. zeae*, *P. pentosaceum*, *P. intermedium*, included in this study, were all adapted to aerobic growth when received. In aerobically incubated stab cultures in tomato agar with glucose, or on lactate agar the growth was well developed throughout the stab after 1–2 days at 30°. When grown on agar plates with free access to air at room temperature, visible growth developed after 1–3 days. For morphological examination the cultures were grown aerobically in stabs and on plates at 20°, employing both of the substrates mentioned above. *P. thoenii* was also grown on G-agar plates in CO₂ atmosphere at 30°. No striking differences were noticed as a result of these different environmental conditions. Short rods of diphtheroid appearance, oval and pear-shaped cells, single, in pairs and in short chains were found as the prevailing forms in all cultures of *P. freudenreichii*, *P. intermedium* and *P. thoenii*. Young cultures of these strains also contained longer cells, which evidently divided by segmentation into the short elements. Short budlike branches were occasionally found in all three species (Pl. 4, figs. 24–26). *P. pentosaceum* grew as irregular, branched rods, which was the dominating form even after 5 days' incubation in surface cultures as well as in stabs (Pl. 4, fig. 27). *P. zeae* had a distinct growth cycle. After 24 hr. on lactate agar the culture contained irregular rods (Pl. 4, fig. 28) which continuously became shorter during the incubation. After 5 days the culture contained only short oval organisms (Pl. 4, fig. 29). On tomato agar with glucose the segmentation was delayed and the long rods prevailed in stabs and in surface cultures even after 5 days (Pl. 4, fig. 30).

Butyribacterium rettgeri. In the 7th edition of *Bergey's Manual* this species is listed as the single representative for the second genus of the new family Propionibacteriaceae. The morphology is described as follows by Barker & Haas (1944): 'Straight or slightly bent, non-capsulated, non-motile rods, $0.7 \times 2-3 \mu$, occurring singly, in pairs, and short chains; branched cells not observed but swollen and clubbed cells occasionally formed. Gram positive.' For morphological examination *B. rettgeri* was grown as stab culture in CO₂ atmosphere at 37° in tomato agar, tomato agar with glucose, G-agar and lactate agar. On the two first-mentioned media visible growth developed within 18 hr., on G-agar and on lactate agar somewhat later. The morphology on these

latter substrates was in good agreement with the cited description (Pl. 5, fig. 31). On tomato agar and tomato agar with glucose *B. rettgeri* showed a more variable morphology (Pl. 5, fig. 32). The length of the rods varied from 2 to 12 μ , branchings were common and of two types: bifurcations as seen on Pl. 5, fig. 32, and asymmetrically located lateral rounded buds (Pl. 5, fig. 33). Cell-wall staining showed that the bifid structures were true branches (Pl. 5, fig. 34), the rods were unicellular or divided by one septum into two cells. The morphological picture was the same throughout the incubation (7 days), and thus the existence of a growth cycle was not indicated.

Lactobacillus. The lactobacilli were grown aerobically on tomato agar either as stabs or as surface cultures. *L. fermenti* (3 strains) was morphologically uniform. The short straight rods consisted of one or two cells as described by Davis, Bisset & Hale (1955); (Pl. 6, fig. 35). The two *L. helveticus* strains contained long rods with a few irregular cross-walls. The rods were often arranged in long curved chains (Pl. 6, fig. 36). This morphology is described as typical for *L. bulgaricus*—*L. acidophilus* by Davis *et al.* (1955). The *L. bulgaricus* isolate was identified on grounds of lacking volatile acids in the fermentation products of milk cultures, ability to grow at 45°, 48° and 52° but not at 15°, and inability to grow in the presence of 2% NaCl or 2% Na taurocholate (Wheater, 1955). The morphology of this *L. bulgaricus* strain was extremely heterogeneous. In stained preparations the rods varied from short, curved cells in pairs (Pl. 6, fig. 37), similar to those described by Davis *et al.* (1955) as typical for the *L. casei-helveticus* group, to long, sinuous chains (Pl. 6, fig. 38). The short curved rods were fragments of long, twisted spirals, easily detected in water mounts (Pl. 6, fig. 39). As seen in Pl. 6, figs 37 and 40, branched rods were found. The short branchings developed from subterminal or lateral pointed buds, similar to the buds of *Corynebacterium acnes*. Both true and false branchings were seen.

DISCUSSION

The scope of the present investigation was twofold: to settle whether the types of bifid bacteria described by Dehnert (1957) and Gyllenberg & Carlberg (1958*b*), can be identified on a morphological basis, and to find out which of the other groups included, all considered to be related to the bifid bacteria, shows the closest morphological resemblance to these organisms.

As to the first point it can be questioned whether a morphological separation of a group as pleomorphic as that of the bifid bacteria is of any taxonomic value. The ordinary descriptions of cell morphology, including information about dimensions of cells and about cell forms, whether rods or cocci, etc., as generally applied to the genera in the order Eubacteriales, cannot without reservations be used for the bifid bacteria. Because of swellings, variation in cell thickness is so large that figures which cover all the organisms of one sub-type at the same time will cover all bifid bacteria. The same is true for the length of the organisms. Further, the morphology shows marked differences on different substrates (cf. Petuely & Eichler, 1954; Dehnert, 1957; Sundman &

af Björkstén, 1958). In the present work the complex bifurcations, known from the literature as typical for the bifid bacteria, and illustrated in Pl. 1, fig. 1, were for instance found only on tomato agar among the strains of types A and B₁, whereas the same cultures grew as prevalently unbranched rods on G-agar. Every morphological description of bifid bacteria should thus be accompanied by a detailed description of the actual cultural conditions. When, however, the morphological examination of the bifid bacteria was performed on cultures grown on a suitable substrate, and the main interest was paid to a few typical configurations and cell forms, in addition to the occurrence of cross-walls, it was found that the morphological differences were big enough to allow a separation.

Our type A of the bifid bacteria (the specific type from breast-fed infants) was not found to possess such striking type-specifying morphological characters as the other types. The optically dense granule seen in the swollen ends of the rods of type A, was not as evident as the stout cell form and the knob-like bifurcations of type B₁, the horn-like branches of type B₂, or the amphora-like organisms of type C. Type A showed morphological resemblances to type B₁ (morphological response to growth on tomato agar) as well as to type B₂ (terminal swellings of the rods) but consisted of septated rods, whereas the organisms of the types B₁ and B₂ were unicellular or contained one central cross-wall. Morphologically the type C (rough form of *Lactobacillus bifidus* var. *pennsylvanicus*) was in several respects different from the other bifid bacteria investigated. The occurrence of slender occasionally filamentous rods, and of club-shaped peculiar amphora-like cells bears resemblance to the morphology of the *Actinomyces bovis* cultures included.

As to the morphological similarity of the bifid bacteria and the related species, the question arises whether all the types of the bifid bacteria should be considered as an entity. The resemblance between the types A and B₁ and *Butyribacterium rettgeri* on one side and between type C and *Actinomyces bovis* on the other side, proved to be at least as close as the internal similarities between these types of the bifid bacteria. The bifurcated rods observed in cultures of *B. rettgeri* on tomato agar were similar to the corresponding forms found in types A and B₁ of the bifid bacteria. The lateral buds seen in *B. rettgeri* were similar in shape and location to those found in type A, isolate K17. The unbranched somewhat irregular rods of *B. rettgeri* were similar in shape to the rod form of type B₁ of the bifid bacteria. A further common feature was the morphological response to the growth environment; the tomato agar induced branchings in the bifid bacteria as well as in *B. rettgeri*. On the other side, the resemblance between the type C and *A. bovis* was not confined to the shape of the cells only. Roughness of growth, poor stainability, and blurred appearance of the cell-wall stained organisms were similar in these cultures. The similarity between the bifid bacteria and the actinomycetes has been pointed out by several authors (e.g. Puntoni, 1937; Negroni & Fischer, 1944; Frank & Skinner, 1954). Unfortunately, their descriptions of the bifid strains in question are not detailed enough to allow the settlement whether type C organisms was dealt with.

The description of *Butyribacterium rettgeri* in the 7th edition of *Bergey's Manual* (1957) obviously needs an addition. The occurrence of branched cells, with true branches as the main structure, was clearly proved in the present work.

As already mentioned in connexion with the description of *Corynebacterium acnes*, the morphological differences between this species and the bifid bacteria seemed to be clear enough to exclude any closer relationship. As a remark on the work of Douglas & Gunter (1946), who placed *C. acnes* in the genus *Propionibacterium*, we wish to state that the morphological criteria found in the present work do not favour such a transfer.

The propionic acid bacteria included in this work formed a morphologically well defined group. The rod forms occasionally found in *Propionibacterium zeae* were similar to rods observed in type B₁ of the bifid bacteria; but the segmentation of the propionic acid bacteria into oval and pear-shaped coccoids is a morphological feature which separates this genus from the bifid bacteria. A common feature of the investigated propionic acid bacteria was the uniform constant thickness of the cells which was independent of the observed variation in length. The bifid bacteria, on the contrary, showed remarkable variations in thickness.

The hitherto unsatisfactory taxonomic position of the bifid bacteria within the genus *Lactobacillus* has given rise to much confusion, and it should be pointed out here that even a superficial microscopical examination will suffice to separate the bifid bacteria from the other lactobacilli. The outer cell walls of the lactobacilli are strictly parallel. Even when strongly curved rods are found, these are throughout even in thickness. The more or less pronounced swellings and irregularities of the rods, always seen in preparations of bifid bacteria, are not found in the lactobacilli. Y- and T-shaped branchings do not *per se* indicate bifid bacteria since, as demonstrated in this report, branching lactobacilli may occur. The differences between lactobacilli and bifid bacteria are most easily seen with the aid of the phase contrast microscope in water mounts or in crystal violet mounts with bright field illumination.

If the taxonomic position of the bifid bacteria is chosen solely on the basis of the morphological relationships within the Gram-positive polymorphic bacteria studied by the present authors, it seems most logical to place them close to the genus *Butyribacterium* (i.e. in the family Propionibacteriaceae as described in the 7th edition of *Bergey's Manual*). As pointed out above, type C (*Lactobacillus bifidus* var. *pennsylvanicus*) makes an exception in this respect and seems to be more closely related to Actinomyces. More information, concerning also physiological and other properties, is needed, however, to permit the decision whether this organism should in fact be separated from the other bifid bacteria.

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EXPLANATION OF PLATES

All plates at $\times 2500$. ph.c. = phase contrast. cr.v.m. = crystal violet mount.

PLATE 1

Bifid bacteria of type A (figs. 1–5) and type B₁ (figs. 6–9), grown at 37° in CO₂-atmosphere.

- Fig. 1. Strain M1. Branched appearance on tomato agar. 3 days. ph.c.
- Fig. 2. Strain M1. Unbranched appearance on G-agar. 3 days. ph.c.
- Fig. 3. Strain D1. G-agar. 3 days. ph.c.
- Fig. 4. Strain K17. G-agar. 24 hr. ph.c.
- Fig. 5. Same as fig. 4. Tannic acid + crystal violet.
- Fig. 6. Strain A4. G-agar. 24 hr. ph.c.
- Fig. 7. Strain A6. G-agar 3 days. ph.c.
- Fig. 8. Strain A2. G-agar. 2 days. Tannic acid + crystal violet.
- Fig. 9. Same as fig. 8.

PLATE 2

Bifid bacteria of type B₂ (figs. 10–12) and type C (figs. 13–16). Grown on G-agar at 37° in CO₂ atmosphere.

- Fig. 10. Strain B18. 4 days. ph.c.
- Fig. 11. Strain C25. 4 days. ph.c. Organisms appearing too big, while the water mount tended to dry.
- Fig. 12. Strain B18. 24 hr. Tannic acid + crystal violet.
- Fig. 13. Strain B4. 24 hr. ph.c.
- Fig. 14. As fig. 13. 2 days.
- Fig. 15. As fig. 13. Tannic acid + crystal violet.
- Fig. 16. As fig. 15.

PLATE 3

Corynebacterium acnes (figs. 17–19) and *Actinomyces bovis* (figs. 20–23), grown at 37° in CO₂-atmosphere.

- Fig. 17. Strain R68. G-agar. 4 days. ph.c.
- Fig. 18. As fig. 17, 15 hr.
- Fig. 19. As fig. 18.
- Fig. 20. Strain A5, CYE substrate, 24 hr. Phosphomolybdic acid–crystal violet. Commensal micrococci seen at lower right. Note difference in stainability of cell walls of the cocci and those of the actinomycete.
- Fig. 21. As fig. 20. 2 days.
- Fig. 22. As fig. 20. 5 days.
- Fig. 23. As fig. 20. 3 days.

PLATE 4

Propionic acid bacteria. Grown at 20° with free access to the air. cr.v.m.

Fig. 24. *P. freudenreichii*. Lactate agar. 4 days.

Fig. 25. *P. thoenii*. Lactate agar. 3 days.

Fig. 26. *P. intermedium*. Lactate agar. 24 hr.

Fig. 27. *P. pentosaceum*. Lactate agar. 4 days.

Fig. 28. *P. xae*. Lactate agar. 24 hr.

Fig. 29. As fig. 28. 4 days.

Fig. 30. As fig. 29. Tomato agar.

PLATE 5

Butyribacterium rettgeri grown at 37° in CO₂ atmosphere.

Fig. 31. G-agar. 3 days. cr.v.m.

Fig. 32. Tomato agar. 3 days. cr.v.m.

Fig. 33. Tomato agar. 4 days. cr.v.m.

Fig. 34. As fig. 33. Phosphomolybdic acid-crystal violet.

PLATE 6

Lactobacilli grown at 37° on tomato agar with free access to the air.

Fig. 35. *Lactobacillus fermenti*, strain BS21. 3 days. Phosphomolybdic acid-crystal violet.

Fig. 36. *L. helveticus*, strain L4. 3 days. Phosphomolybdic acid-crystal violet.

Fig. 37. *L. bulgaricus*. 2 days. Phosphomolybdic acid-crystal violet.

Fig. 38. As fig. 37. 24 hr.

Fig. 39. As fig. 37. 6 days. cr.v.m.

Fig. 40. As fig. 37.

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V. SUNDMAN, K. AF BJÖRKSTEN & H. G. GYLLENBERG—MORPHOLOGY OF BIFID BACTERIA. PLATE I

(Facing p. 384)

V. SUNDMAN, K. AF BJÖRKSTEN & H. G. GYLLENBERG—MORPHOLOGY OF BIFID BACTERIA. PLATE 2

V. SUNDMAN, K. AF BJÖRKSTEN & H. G. GYLLENBERG—MORPHOLOGY OF BIFID BACTERIA. PLATE 3

V. SUNDMAN, K. AF BJÖRKSTEN & H. G. GYLLENBERG—MORPHOLOGY OF BIFID BACTERIA. PLATE 4

V. SUNDMAN, K. AF BJÖRKSTEN & H. G. GYLLENBERG—MORPHOLOGY OF BIFID BACTERIA. PLATE 5

V. SUNDMAN, K. AF BJÖRKSTEN & H. G. GYLLENBERG—MORPHOLOGY OF BIFID BACTERIA. PLATE 6