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Numerical Taxonomy of Certain Coryneform Bacteria[1]

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ABSTRACT

DA SILVA, G. A. NIGEL (Iowa State University, Ames), AND JOHN G. HOLT. Numerical taxonomy of certain coryneform bacteria. J. Bacteriol. 90:921-927. 1965—An electronic computer was used to sort 32 strains of coryneforms into groups with the tree-sort program. The similarity values obtained in this procedure were then used to construct a dendrogram depicting the phenetic resemblance among the taxa. The results indicated that all the phytopathogens studied were sufficiently distinct from the type species, *Corynebacterium diphtheriae*, to be excluded from the genus *Corynebacterium*. The grouping of some of the phytopathogens with *Microbacterium lacticum* is discussed. *C. fascians* appeared so distinct from the other strains studied that it should probably be excluded from the Corynebacteriaceae. The phenetic resemblance of *Brevibacterium linens* and *Arthrobacter globiformis* was emphasized, and the new combination, *Arthrobacter linens*, was proposed. In addition, because of distinct dissimilarity from the type species, it would seem desirable to exclude *Arthrobacter tumescens* from the genus *Arthrobacter*. The justification for classifying *Kurthia zopfii* in a family separate from the Corynebacteriaceae would appear to be open to serious question. It was concluded that the present taxonomic positions of *Listeria monocytogenes*, *Cellulomonas biazotea*, and *Cellulomonas fimi* are satisfactory.

"There are perhaps few groups of bacteria of which the typical representatives are easier to recognize and the aberrant types more numerous and more difficult to separate than those which originally constituted the genus *Corynebacterium*" (Jensen 1952). When first introduced by Lehmann and Neumann (1907) to accommodate the diphtheria bacillus and certain similar parasitic organisms, the genus was a pleasingly simple one. Its species were characterized by gram-positive, nonsporeforming, nonmotile, nonacid-fast rods which showed a tendency to irregular staining. During the nearly six decades since its introduction, however, so many different club-shaped bacteria have been included in the genus, and its outlines have become so fluid, that many workers have had recourse to protest against what Conn and Dimmick (1947) refer to as the "misuse of the term *Corynebacterium*."

In recent years, the word "coryneform" has come to be used for a wide variety of gram-positive, nonsporeforming rods. Etymologically, however, the word coryneform means clublike. Originally, this vernacular designation was used with reference to those species belonging to the genus *Corynebacterium*. In many instances, modern usage of this word would appear to be inconsistent with the implied meaning. For the purpose of this paper, however, coryneform is used in the following sense: gram-positive, nonsporeforming, nonacid-fast rods showing metachromatic granules; many are club-shaped and exhibit characteristic palisade arrangement of cells; many exhibit morphogenesis and a tendency to irregular staining.

The classification of these organisms has always been beset with problems. Probably the most controversial aspect, however, centers around the inclusion of 11 plant pathogens in the genus *Corynebacterium*. To the plant pathologist, these organisms are relatively unimportant. Yet, to the bacterial systematist, there has been a constant problem as to what genus or genera they should occupy. *C. sepedonicum*, for example, the potato ring rot bacterium, has been placed in six different genera since its isolation in 1913. In spite of this unsettled state of classification, few comprehensive studies have been made on the relationship of the plant pathogens to the other coryneforms.

[1] Based on a thesis submitted by the senior author to the Graduate College of Iowa State University in partial fulfillment of the requirements for the M.S. degree.

The coryneform bacteria are widespread in nature. Some of the soil forms have been well studied for their ability to degrade cellulose, others for their ability to undergo morphogenesis. Their taxonomy, however, has been sadly neglected. Consequently, not unlike their plant pathogenic counterparts, their generic placement has been quite controversial.

Certain gram-positive bacteria closely resembling the soil coryneforms are now classified in the family Brevibacteriaceae (*Bergey's Manual*). Some of these organisms, now considered as belonging to the genus *Brevibacterium*, have been investigated in some detail as a result of investigations of the microbial synthesis of glutamic acid. Mulder and Antheunisse (1963) found that the type species, *B. linens*, was quite similar to *Arthrobacter globiformis*. This tends to question the justification for classifying these two organisms in two separate families.

This investigation was undertaken primarily to determine, by use of the methods of numerical taxonomy, the relationship of the coryneform plant pathogens to *C. diphtheriae* and to the type species of six other genera of coryneforms.

MATERIALS AND METHODS

Organisms. A total of 32 bacterial strains was studied. The strain designations and sources of these cultures are listed in Table 1. Cultures were maintained on a medium consisting of Tryptic Soy agar (Difco) supplemented with 0.4% yeast extract.

Determination of properties. The 57 standard morphological and physiological properties listed in Table 2 were determined for each of the organisms. The procedures employed in the determination of all properties, except numbers 1, 5, 6, 13, 14, 18, 23, and 57, were according to standard methods (Society of American Bacteriologists, 1957). Property 1 was determined by the method of Leifson (1960). Properties 5 and 6 were determined by direct microscopic observation of the bacterial growth on an agar plate as follows. A young broth culture of the species being examined was streaked onto an agar plate and incubated,

TABLE 1. *Bacterial strains used in the computer analysis**

Family	Genus	Species
Corynebacteriaceae	*Corynebacterium*	*C. diphtheriae* (type species) ATCC 11913
		C. fascians ATCC 12974, 12975
		C. flaccumfaciens ATCC 6887, 7392
		C. insidiosum NCIB 83, ATCC 10253
		C. michiganense ATCC 4450, 10202
		C. poinsettiae ATCC 9069, 9682
		C. sepedonicum ATCC 9850
		C. tritici ATCC 11402, 11403
		Bean wilt bacterium†
	Arthrobacter	*A. globiformis* (type species) ATCC 8010, NCIB 8620, 8907
		A. tumescens MRI 836, ATCC 6947
	Cellulomonas	*C. biazotea* (type species) ATCC 486, NCIB 8077
		C. fimi ATCC 484
	Microbacterium	*M. lacticum* (type species) ATCC 8180, 8181
	Listeria	*L. monocytogenes* (type species) ATCC 4428, 15313 (type strain)
Brevibacteriaceae	*Brevibacterium*	*B. linens* (type species) ATCC 9172, 9174, 9175
	Kurthia	*K. zopfii* (type species) ATCC 6900, 10538

* The systematic arrangement is according to *Bergey's Manual*. Abbreviations: ATCC = American Type Culture Collection, NCIB = National Collection of Industrial Bacteria (Great Britain), MRI = Microbiology Research Institute, Ottawa, Canada.

† Acquired from J. Dunleavy, Department of Botany and Plant Pathology, Iowa State University.

TABLE 2. *Properties used in the characterization of the organisms studied*

Properties		
1. Motility	21. Urea-positive	41. Acid from raffinose
2. Hemolysis on blood-agar	22. Cellulose digestion	42. Acid from rhamnose
3. Presence of capsule	23. Ammonia from peptone	43. Acid from salicin
4. Metachromatic granules	24. Methyl red-positive	44. Acid from sorbitol
5. Snapping division	25. Hydrogen sulfide produced	45. Acid from sorbose
6. Morphogenesis	26. Resists 62 C for 10 min	46. Acid from sucrose
7. Turbidity in broth	27. Indole produced	47. Acid from trehalose
8. Sediment in broth	28. Growth at 37 C	48. Acid from xylose
9. Reduction of litmus milk	29. Acid from adonitol*	49. Surface growth in broth
10. Reduction of tellurite	30. Acid from arabinose	50. Colony elevation
11. Nitrates reduced	31. Acid from cellibiose	51. Colony edge
12. Casein hydrolyzed	32. Acid from fructose	52. Growth form on a slant
13. Tributyrin hydrolyzed	33. Acid from galactose	53. Surface texture of colony
14. Tyrosine hydrolyzed	34. Acid from glucose	54. Pigmentation
15. Starch hydrolyzed	35. Acid from inositol	55. Growth in NaCl at concentrations of 2.5, 5, 7.5, 10, and 12.5%
16. Gelatin hydrolyzed	36. Acid from lactose	
17. Voges-Proskauer-positive	37. Acid from maltose	
18. Cytochrome oxidase-positive	38. Acid from mannitol	56. Gram stain
19. Catalase-positive	39. Acid from mannose	57. Average length of the cell
20. Citrate-positive	40. Acid from melibiose	

* The basal medium employed in determining properties 29 to 48 for all species except *Corynebacterium diphtheriae* was nutrient broth. Hiss serum water was used for *C. diphtheriae*. The method of preparation of the Hiss serum water was as outlined by Wilson and Miles (1964).

after which a sterile cover slip was placed on top of the growth and examined directly with a phase microscope. For property 5, cultures were examined at intervals of 4, 6, and 10 hr. For property 57, determinations were made with the aid of a micrometer on the 24-hr growth used for determining the previous property. The presence of cytochrome oxidase was determined by flooding 24- to 48-hr-old agar plate cultures with a 1% solution of *p*-aminodimethylaniline oxalate. Development of a pink colony which turned maroon and finally black was considered to be positive. Production of ammonia from peptone was determined in accordance with the method outlined by Dowson (1957). Hydrolysis of tyrosine and tributyrin were determined by use of the method of Davis and Park (1962).

Coding of features for data analysis. A code symbol (A, B, C, D, or E) was assigned for each alternative state in which a property could occur. Since properties 1 to 48 existed as one of two mutually exclusive states, they were coded for the computer analysis as A = positive, B = negative. For properties 50 to 55, the method suggested by Lockhart and Hartman (1963) was used. A different symbol was assigned to each separate state in which a property existed.

Many of the strains, particularly the soil coryneforms, exhibited variability in their gram reaction, depending on the age of the culture at which the stain was made. The method employed in coding this feature is as follows. When the reaction to the Gram stain was positive at 24 and 72 hr, code symbol A was assigned; when the reaction was gram-negative at 24 hr and gram-positive at 72 hr, B was assigned; and when the reaction was gram-negative at 24 and 72 hr, code C was assigned. Property 49 was coded according to the method of Lockhart (1964). The presence of surface growth was considered as a primary property; the presence of a ring or membrane, as a secondary property. For property 57, all strains with an average cell length less than 3.0 μ were assigned one code symbol; those in excess of 3.0 μ were assigned an alternative symbol.

Computer operation. The machine employed in the analysis of these data was the IBM 7074 of the Iowa State University Computation Center. The computer method used was one developed by Lockhart, Hartman, and Smith in 1963 (*unpublished data*), and is known as the tree-sort method. In this method, the coded properties were transcribed onto special data cards and then supplied to the computer. The machine searches the data for those pairs or groups of strains which are alike in all their properties, and then prints up these clusters of strains as forming a group. The computer next searches the data for those strains which differ in only one property and prints up these. This procedure is repeated over and over, the machine incrementing the number of differences by one each time until the taxa are all grouped together in one large group. In this way, the strains form several smaller clusters separated at varying difference levels which eventually join to form one large cluster at sufficiently large levels of difference. In the tree-sort method, there is a direct linear relationship between the difference level and the percentage similarity. [Conversion of the difference level to percentage

similarity was obtained according to the formula: $S_{ij} = (N_F - ND_{ij})/N_F$, where S_{ij} = percentage similarity between an ith and jth taxon, N_F = total number of features represented per taxon, ND_{ij} = number of features by which the ith and jth taxon differed.] Since the latter criterion is a more convenient way of expressing phenetic resemblance among the taxa, it was used for presentation of the results.

RESULTS AND DISCUSSION

The results of the analyses were arranged in the form of a dendrogram, which is represented in Fig. 1.

From the dendrogram it is evident that at least four distinct clusters emerge. The largest of these clusters is delimited by a phenon level of 82%, and contains five of the phytopathogenic species, *C. poinsettiae*, *C. tritici*, *C. sepedonicum*, *C. insidiosum*, and *C. michiganense*. Cluster formation revolves around *M. lacticum*. The phenetic resemblance among this cluster appears to be reasonably convincing. However, it is possible that this resemblance is not entirely correct and may have resulted from the fact that *Microbacterium lacticum* was incompletely compared with the phytopathogens. One aspect of

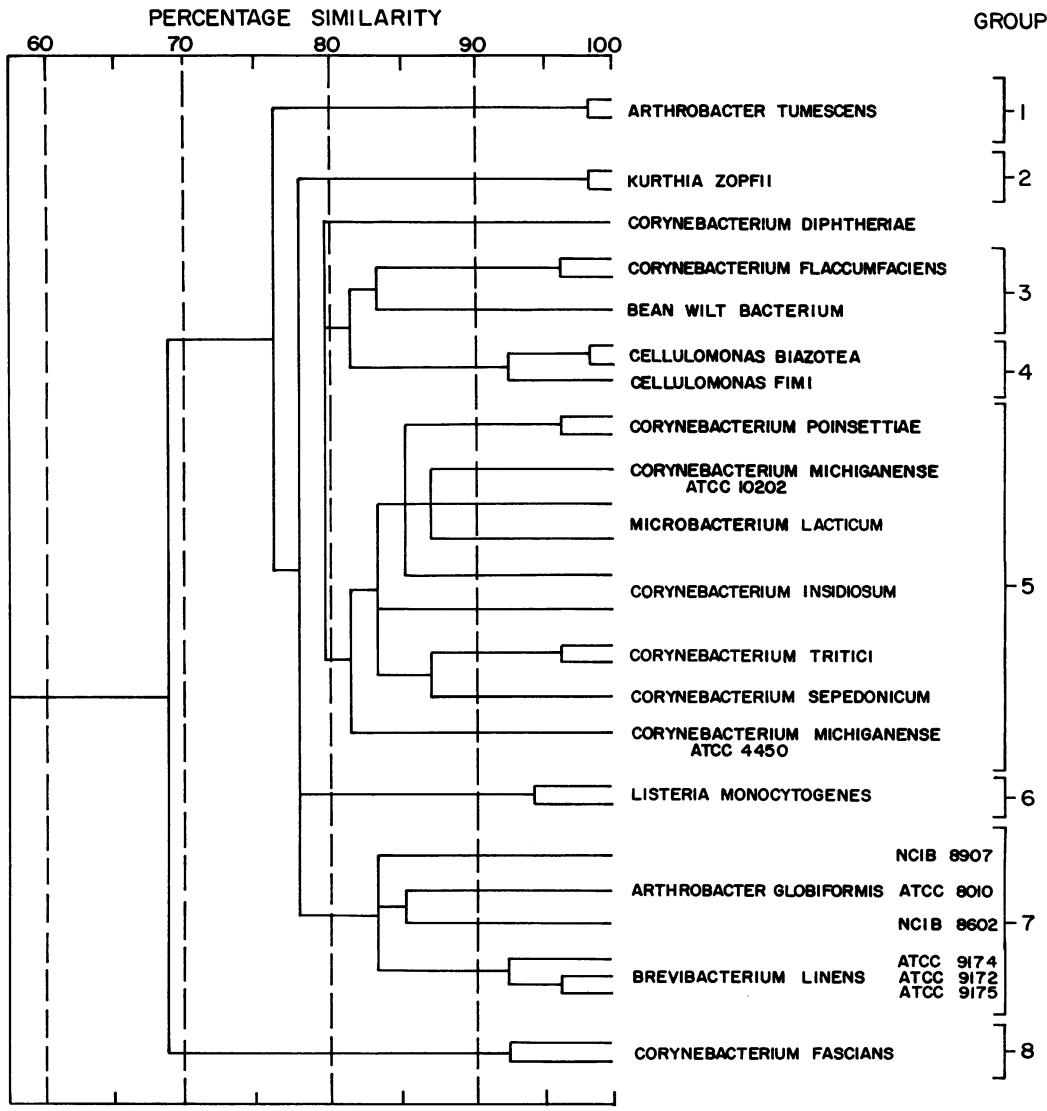

FIG. 1. *Dendrogram depicting the phenetic resemblance among the species studied. The horizontal lines represent the taxa and the levels at which they unite to form groups. The broken vertical lines indicate the phenon levels.*

this incomplete comparison involves the pathways for glucose metabolism. Most of the coryneform phytopathogens appear to metabolize glucose oxidatively (Katznelson, 1958; Starr, 1959). On the other hand, *M. lacticum* appears to use the Embden-Meyerhof pathway. Surely such differences in enzymatic constitution should be taken into consideration when computing phenetic resemblance. However, it is difficult to assess the extent to which this property should be weighted. Consequently, it was not used in determining these similarity values.

The second largest cluster is made up of strains of *A. globiformis* and *B. linens*. The cluster is delimited by a phenon level of approximately 83%. It appears as the most distinct cluster of the organisms studied, and, in spite of the fact that the two constituent species each belong to different families, the grouping is not altogether surprising. Other workers have also reported that these two species appear to be phenetically similar (Mulder and Antheunisse, 1963). It is surprising to find, however, that *A. tumescens* is so distinct from its type species, *A. globiformis*. It can be seen that this species has a phenon level, in relation to the other species, of just over 75%.

Several species in the genus *Arthrobacter* appear to be out of place. Cummins (1962) and Cummins and Harris (1958, 1959) pointed out that major discrepancies exist between the cell-wall constituents of *A. globiformis* and *A. tumescens*. Zagallo and Wang (1962) presented evidence of significant metabolic dissimilarity among the species in this genus. They found, for example, that the type species used mainly the Embden-Meyerhof pathway for the metabolism of glucose, but that other species—*A. simplex*, *A. pascens*, and *A. atrocyaneus*—relied primarily on the intermediary formation of gluconate.

The tendency in the past few years has been to assign species to the genus *Arthrobacter* mainly on the basis of morphology, often despite distinct differences in nutritional requirements (Zagallo and Wang, 1962). This practice is understandable in view of the fact that all too often it is difficult to obtain significant physiological response from these organisms. This has led to the questionable inclusion of many species in this genus. It would seem that our results cast further doubt on the organization of this genus as it stands in *Bergey's Manual*. Clearly, on the basis of the comparative relationships presented in Fig. 1, *A. tumescens* no more belongs in the genus *Arthrobacter* than does *Listeria monocytogenes* or, for that matter, *C. diphtheriae*.

The genus *Brevibacterium* has similarly become the repository for a variety of gram-positive bacteria. The species in this genus range from physiologically inert to active fermentative and oxidative organisms. Indeed, the only thing definite about the generic description in *Bergey's Manual* is that the organisms are gram-positive, nonsporeforming, unbranched rods. It has been pointed out previously that *B. linens* possesses many of the characteristics peculiar to coryneforms. In addition, Su and Yamada (1960) have called attention to the fact that this species is quite different physiologically from most of the other species in the genus. In view of this evidence, and because our analysis clearly shows the closeness of *B. linens* to *A. globiformis*, we propose that *B. linens* be transferred to the genus *Arthrobacter* and the new combination, *Arthrobacter linens* (Wolff) da Silva and Holt, be created. It will be necessary to study the other species in the genus *Brevibacterium* to determine their taxonomic position. Such studies are in progress at this time, and it is hoped that the reallocation of the remaining species will soon be forthcoming.

The next largest clusters consist of three plant pathogens and three cellulolytic coryneforms. These phytopathogens, which are represented by *C. flaccumfaciens* and an unnamed strain causing soybean wilt, appear to be distinct from the group of phytopathogens previously discussed. They could probably be considered as belonging to a separate genus. The *Cellulomonas* strains appear to be distinct. This is a welcome finding, since more often than not the sole criterion used in assigning organisms to this genus is their ability to attack cellulose. It would thus appear that, as far as these species of *Cellulomonas* are concerned, the ability to degrade cellulose is sufficiently highly correlated with other features to make it a reliable diagnostic feature with which to separate these organisms from the other coryneforms.

The species *Corynebacterium fascians* was the most distinct of all the species studied. It had a phenon level of less than 70%, indicating marked difference from the other coryneforms. Several workers (Starr et al., 1943; Conn and Dimmick, 1947) have expressed the opinion that it does not belong in the genus *Corynebacterium*. In fact, Conn and Dimmick considered it to be closely related to *Nocardia*, which would place it in an entirely different order. Cummins (1962) stated that, on the basis of its cell-wall constituents, it more closely resembles *Mycobacterium smegmatis*. Further, Lacey (1955) observed that, by growing *C. fascians* strains on a synthetic medium low in nitrogen, filamentous growth could be obtained which resembled that produced by *Nocardia* species. In addition, under such conditions, most of the strains she studied were acid-fast. On the other hand, evidence in support of its present taxonomic position is not entirely lacking. It has been found that the guanine plus

cytosine ratio is approximately the same in *C. fascians* as it is in *C. diphtheriae* (Marmur, Falkow, and Mandel, 1963). Nevertheless, most evidence points to the fact that there is justification for considering *C. fascians* as being sufficiently distinct to question the desirability of its inclusion in any genus of the Corynebacteriaceae. Hence, in line with previous workers, we are of the opinion that it should be excluded from the genus *Corynebacterium*. However, we are reluctant to propose that it be assigned to a particular taxon, since the appropriate comparative studies are as yet incomplete.

From the analyses, it is evident that the phytopathogens differ significantly from the type of the genus in which they are presently classified. These findings confirm the doubts expressed by previous workers as to the correctness of including these organisms in the genus *Corynebacterium*. Under the circumstances, it would seem desirable that these species be excluded from this genus and allocated to other genera. The disposition of two of these phytopathogenic species has already been discussed. It has been pointed out that, in the case of the third group, additional study may be necessary to confirm the validity of the cluster. However, for the purposes of allocation of the species of this cluster to a different taxon, two type species are available, *M. lacticum* and *C. insidiosum*. The latter name was used by Savulescu (1947) for the type of the genus *Burkholderiella*. Since the name is both validly published and legitimate, it would seem to be appropriate. However, the name *M. lacticum* has priority over *B. insidiosum* by virtue of being proposed earlier. Hence, under the circumstances, the generic name *Burkholderiella* cannot be used for this group of organisms as long as *M. lacticum* is included. On the other hand, for reasons discussed previously, *M. lacticum* would also be undesirable.

In order not to complicate matters further, we are reluctant at this time to suggest that the five species of plant pathogens be placed in a separate genus. However, we wish to emphasize the need for further study aimed at removing these species from the genus *Corynebacterium* and placing them in appropriate new taxa.

The present taxonomic rank assigned to *Kurthia* and *Listeria* (*Bergey's Manual*), as reflected by the strains of these genera studied, would appear to be satisfactory. Their taxonomic position in relation to each other appears less satisfactory. This is evidenced by the fact that both *K. zopfii* and *L. monocytogenes* have the same phenon level in relation to the other strains, and yet they are classified in separate families. On the basis of our findings, there seems to be little justification for placing the genus *Kurthia* in a family separate from the Corynebacteriaceae.

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