A Numerical Taxonomical Study of Some *Corynebacteria* and Related Organisms

By B. J. HARRINGTON*

Department of Virology and Bacteriology, University of Birmingham

SUMMARY

A numerical Adansonian analysis has been performed on the results of a wide range of biochemical and physiological characters of organisms at present classified in the genera *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Arthrobacter* and *Jensenia*. The results show that some of the organisms need to be reclassified. *Corynebacterium ulcerans* could be considered as another type of *C. diphtheriae*, and *C. pyogenes* and *C. haemolyticum* have little similarity to any of the other corynebacteria. *Corynebacterium equi* and *Jensenia canicruria* appear to be closely related to the mycobacteria examined. The plant pathogens *C. rathayi*, *C. betae* and *C. michiganense* seem to be distinct from most of the animal parasites in this genus. The mycobacteria, nocardia and most of the corynebacteria from animal sources form one large group at the 51 % similarity level. Acid-fastness does not seem to be a good criterion for separating the genus *Corynebacterium* from the genus *Mycobacterium*, some of the corynebacteria showed varying degrees of acid-fastness.

INTRODUCTION

In the genus *Corynebacterium*, *Bergey's Manual* (1957) lists thirty-three species. In addition to these there are others which have been described, and some which have been transferred from this genus to other genera in *Bergey's Manual* or by other authors, but which are still retained as corynebacteria in culture collections and by some authors. Several workers have suggested that the genus *Corynebacterium* contains some species, particularly those from plant and soil sources, which should not be there (Conn, 1947; Conn & Dimmick, 1947; Clark, 1952; Cummins, 1962*a*; da Silva & Holt, 1965). Cummins (1962*b*) and Perkins & Cummins (1964) found that most of the plant pathogens they examined had markedly different cell-wall compositions from those of the animal parasites in the genus. Harrington (unpublished) has found that *C. michiganense* has a cell-wall amino acid composition similar to those of most of the other plant pathogens, while the soil strain *C. fimi* has the amino acid pattern of the animal parasites.

Not only is there confusion within the genus *Corynebacterium*, but also disagreement as to its taxonomic position in relation to other genera, notably *Mycobacterium* and *Nocardia*. Mainly on the basis of acid-fastness, *Bergey's Manual* (1948) transferred the genus *Corynebacterium* to a new family, Corynebacteriaceae, in the *Eubacteriales*, the genera *Mycobacterium* and *Nocardia* remaining in the Actinomycetales. The seventh edition of *Bergey's Manual* (1957) continued this division, although Bisset & Moore (1949), Jensen (1953) and others had pointed out that

* Present address: Department of Bacteriology, University of Wisconsin, Madison, Wis., 53706, U.S.A.

morphologically these three genera are closely related and not always distinct from each other. Cummins & Harris (1958) found that these genera have almost identical patterns of cell-wall components, and Kwapinski (1956, 1964) and Cummins (1962*b*) have shown serological relationships between members of these genera.

This present work includes an Adansonian numerical study of the taxonomy of some species currently classified as corynebacteria, and of the relationships of these organisms to others in some related genera.

METHODS

Strains examined. Table 1 shows the origins of the strains used in this study. Those received from culture collections have the names they bore when they were received, those from the Department's collection have the names under which they are maintained.

Table 1. *The strains used and their sources*

From the National Collection of Type Cultures

<i>Corynebacterium equi</i>	1621	<i>C. diphtheriae gravis</i>	7233
<i>C. diphtheriae</i> P.W. 8	380	<i>C. hofmannii</i>	231
<i>C. renale</i>	7448	<i>C. bovis</i>	3224
<i>C. flavidum</i>	746	<i>C. xerosis</i>	7243
<i>C. ulcerans</i>	8640	<i>C. ovis</i>	4631
<i>C. segmentosum</i>	934	<i>C. murium</i>	949
<i>C. fimi</i>	7547	<i>C. viscosum</i>	2416
<i>C. haemolyticum</i>	8452	<i>C. pyogenes</i>	9825

From the National Collection of Plant Pathogenic Bacteria

<i>C. fascians</i>	764	<i>C. betae</i>	372
<i>C. rathayi</i>	797	<i>C. michiganense</i>	382

From the National Collection of Dairy Organisms

<i>C. helvolum</i>	1192	<i>Arthrobacter</i> sp.	1233
<i>Arthrobacter</i> sp.	1234		

From the Department's collection

<i>C. diphtheriae intermedius</i>	<i>C. diphtheriae mitis</i>
<i>C. hofmannii</i>	<i>Mycobacterium phlei</i>
<i>M. lacticola</i>	<i>M. smegmatis</i>
<i>M. butyricum</i>	<i>Jensenia canicruria</i>

Isolated by the author

<i>Nocardia</i> sp. from human saliva
<i>Nocardia</i> sp. from soil

Strain maintenance. The strains were maintained in a medium of the following composition, percentages are w/v. Tryptone (Oxoid L42) 0.5 %, proteose peptone (Oxoid L46) 0.25 %, casein hydrolysate (Allen and Hanbury Ltd., acid hydrolysed, vitamin-free) 0.25 %, NaCl 0.1 %, KH_2PO_4 0.02 %, Na_2HPO_4 0.06 %, MgSO_4 0.001 %, MnCl_2 0.001 %. The pH was adjusted to 7.2. This medium supported good growth of all the strains.

Characters examined. Table 2 shows the characters examined. With the exception of the Voges-Proskauer test, the methyl red test, reactions in litmus milk, growth in Koser citrate, growth on Hoyle medium, and liquefaction of heat-coagulated serum, all the tests were performed using the maintenance medium as the basal nutrient

Table 2. Characters examined

Acid from: *		Methyl red test
Arabinose	Xylose	Voges-Proskauer test
Rhamnose	Glucose	Salicin hydrolysis
Fructose	Mannose	Aesculin hydrolysis
Galactose	Maltose	Starch hydrolysis
Lactose	Sucrose	Reactions in litmus milk
Raffinose	Starch	Oxid. or ferm. of glucose
Inulin	Dextrin	Polysaccharide on medium +
Glycogen	Glycerol	Glucose Sucrose
Erythritol	Adonitol	Intracellular starch on medium +
Mannitol	Dulcitol	Glucose Sucrose
Sorbitol	Salicin	Growth in Koser citrate
Aesculin	Inositol	Acetic acid from ethanol
DHA from glycerol		Calcium lactate oxidation
Gluconate oxidation		Tyrosine breakdown
Lipolysis on:		Xanthine breakdown
Butter	Castor oil	Lysine decarboxylation
Tweens 20, 40, 60 and 80		Ornithine decarboxylation
Glycerol tributyrates		Phenylalanine deamination
Lecithinase production		Sodium malonate utilisation
On egg yolk medium:		Ammonia from arginine
Lipolysis	Proteolysis	Serum hydrolysis
Gelatin hydrolysis		Casein hydrolysis
Coagulated serum hydrolysis		Ammonia from basal medium
Phosphatase production		Urease production
pH in basal medium after 7 days		Nitrite reduction
Nitrate reduction		Oxidase production
Catalase production		Methylene blue reduction
Sodium hippurate hydrolysis		Benzidine/H ₂ O ₂ test
Peroxide production		Haemolysis of horse blood
H ₂ S from basal medium		Motility
H ₂ S from Na ₂ S ₂ O ₃		Anaerobic growth
H ₂ S from cysteine		Reaction to the Gram stain
Resistance to antibiotics:		Tolerance of NaCl:
Polymyxin B	Mycostatin	2.5 % 5.0 % 10.0 %
Tetracycline	Penicillin	Tolerance of potassium tellurite:
Oleandomycin	Novobiocin	0.0175 % 0.035 %
Chlortetracycline	Terramycin	Tolerance of sodium selenite:
Chloramphenicol	Kanamycin	0.0175 % 0.035 %
Erythromycin	Neomycin	Tolerance of triphenyltetrazolium chloride:
Streptomycin	Bacitracin	0.001 % 0.0025 % 0.01 % 0.025 %
Growth on Hoyles medium		Tolerance of sodium taurocholate:
Growth at initial pH values:		2.0 % 5.0 %
5.0 6.0 7.0 8.0 9.0		Growth at:
Pigmentation		5° 25° 30° 37° 45°
Morphology: presence of:		On basal medium, acid-fast to:
Long rods	> 5 µ	H ₂ SO ₄ 1 % 2 % 5 % 10 % 20 %
Medium rods	3 µ-5 µ	On basal medium + 2 % glycerol, acid-fast to:
Short rods	< 3 µ	H ₂ SO ₄ 1 % 2 % 5 % 10 % 20 %
Coccal forms		
Oval forms		
Branching		

* No strain produced gas from any carbohydrate.

medium. The strains from animal sources were incubated at 37°, all the others at 30°. For complete details of the test procedures, see Harrington (1964).

For the numerical analysis, all the characters, with the exceptions listed below, were recorded as positive or negative. Pigmentation was scored as white, creamy

yellow, pink or orange; haemolysis as α , β , or negative; pH of the basal medium after 7 days as no change, raised or lowered; reactions in litmus milk as acid, acid with clot, no change, or alkaline; digestion and reduction of the indicator were scored as positive or negative; pathway of glucose breakdown, where applicable, as oxidation or fermentation. To make the processing of the results, which was done by hand, easier, degrees of positivity were not taken into account. All the characters were given equal weight and the equation proposed by Sneath (1957) was used to obtain the similarity values. This equation is $S = a/(b+c+a)$, where a = the number of tests in which both members of a pair are positive, $b+c$ = the number of tests in which one member is positive and the other negative, and negative matches are not included.

Acid-fastness. Two days' growth on the maintenance medium and on the maintenance medium + 2% glycerol was used. Smears were made on microscope slides and stained for 5 min. with hot strong carbol fuchsin, then washed with water and treated with four changes of sulphuric acid, each application lasting 1.5 min. The acid was washed off the slide with water and the smear blotted dry between each acid change. After the last washing the smears were counter-stained with dilute malachite green. The first concentration of acid used was 1%. Smears were then made of those strains which showed more than approximately 25% of the cells retaining the carbol fuchsin, and these were stained as before, with 2% acid being used to effect decolorization. This process of elimination was repeated with 5% acid, 10% acid and 20% acid until complete or almost complete decolorization was achieved.

RESULTS

Numerical analysis. After the similarity values had been calculated as percentages, a cluster analysis was performed as described by Sneath (1962), a strain being admitted to a group at the highest similarity level it had with any member of that group. Figure 1 is the similarity matrix after the cluster analysis had been performed, and Figure 2 is the dendrogram derived from the similarity matrix.

The main points of interest are:

- (1) The relatively wide range of S values among the strains of *Corynebacterium diphtheriae*.
- (2) The high S values of *Corynebacterium ulcerans* with the 'gravis' and 'mitis' strains of *C. diphtheriae*.
- (3) *Corynebacterium pyogenes* and *C. haemolyticum* have very low S values with all the other strains examined here.
- (4) *Jensenia canicruria* and *Corynebacterium equi* come within the mycobacterium group.
- (5) The plant pathogens with the atypical cell wall patterns, *Corynebacterium rathayi*, *C. betae* and *C. michiganense*, have low S values with most of the corynebacteria from animal sources, and these values are lower than those of the other plant pathogen, *C. fascians*, particularly with the strains of *C. diphtheriae*.
- (6) The mycobacteria, the nocardia and the majority of the animal parasitic corynebacteria are in one group at the 51% S level.

Acid-fastness. Table 3 shows the results of the acid-fast staining of those strains which showed any degree of acid-fastness on either medium. Most of those that

Fig. 2. Dendrogram obtained from the similarity matrix.

Table 3. Acid-fastness of strains grown on different media

Concentration of H ₂ SO ₄	Maintenance medium					Maintenance medium + 2 % glycerol				
	1 %	2 %	5 %	10 %	20 %	1 %	2 %	5 %	10 %	20 %
<i>C. diphtheriae gravis</i>	+	+	-	-	-	+	+	+	-	-
<i>C. diphtheriae mitis</i>	+	-	-	-	-	+	+	+	-	-
<i>C. diphtheriae</i> P.W. 8	+	+	+	-	-	+	+	+	-	-
<i>C. ulcerans</i>	+	-	-	-	-	+	+	+	-	-
<i>C. flavidum</i>	-	-	-	-	-	+	-	-	-	-
<i>C. renale</i>	-	-	-	-	-	+	-	-	-	-
<i>Nocardia</i> sp. (oral)	+	+	-	-	-	+	+	+	+	-
<i>C. hofmannii</i> (231)	+	-	-	-	-	+	-	-	-	-
<i>Nocardia</i> sp. (soil)	+	-	-	-	-	+	-	-	-	-
<i>Jensenia canicruria</i>	+	+	-	-	-	+	+	-	-	-
<i>M. smegmatis</i>	+	+	+	-	-	+	+	+	+	-
<i>M. phlei</i>	+	+	+	-	-	+	+	+	+	-
<i>M. butyricum</i>	+	+	-	-	-	+	+	+	+	-
<i>M. lacticola</i>	+	+	+	-	-	+	+	+	+	-
<i>C. helvolum</i>	-	-	-	-	-	+	-	-	-	-

showed some resistance to decolorisation when grown on the maintenance medium showed an increased resistance when grown on this medium + 2 % glycerol. Even on the glycerol-containing medium all the mycobacteria examined were decolorized by 20 % sulphuric acid, while on this medium *C. ulcerans* and the 'gravis', 'mitis' and P.W.8 strains of *C. diphtheriae* were resistant to 5 % acid.

DISCUSSION

Among the four strains of *Corynebacterium diphtheriae* there is a range of similarity values, from 87 % for the 'gravis + mitis' pairing down to 51 % for the *intermedius* + P.W.8 pairing, the lowest values being those of pairings which include the 'intermedius' strain. In view of this wide range of S values, the position of *C. ulcerans*, which has been found to cause diphtheria-like lesions and produce a similar toxin to that of *C. diphtheriae* (see Wilson & Miles, 1964), is of interest. It was found here that this organism has an S value of 62 % with the 'mitis' strain and 60 % with the 'gravis' strain. The taxonomic rank of *C. ulcerans* is still a matter of some dispute. Saxholm (1951) and Henriksen & Grelland (1952) thought the organisms of the *C. ulcerans* type, the so-called 'diphtheria-like corynebacteria', could either be regarded as a distinct species or as a variety of *C. diphtheriae*. Howard & Jann (1954) felt that their results with bacteriophages lytic for the diphtheria-like corynebacteria showed these organisms to be distinct from *C. diphtheriae*, although two of their diphtheria-like corynebacteria resembled *C. diphtheriae* in that they were resistant to all the phages used. Wilson & Miles (1964) believed that *C. ulcerans* is sufficiently well defined to qualify for species rank, basing their decision on the results of Jebb (1948) who found it to differ from *C. diphtheriae* in only four characters. If the four strains of *C. diphtheriae* examined here are to be regarded as representatives of one species, then on the S values of the 'gravis' and 'mitis' strains with *C. ulcerans* found here, this organism should be reclassified as a variety of *C. diphtheriae*.

Corynebacterium pyogenes and *C. haemolyticum* were found by Cummins & Harris (1956) to have cell-wall amino acid and sugar patterns characteristic of the streptococci, not the corynebacteria, and they suggested that these organisms should be reclassified as streptococci. The similarity values of these two strains with all the other strains are low, which supports the hypothesis that they are not corynebacteria. Sneath & Cowan (1958), in their numerical taxonomical study of a wide range of bacteria, found that *C. pyogenes* fell in the group which contained the streptococci.

There is a distinct group at the 61 % level made up of the mycobacteria, *Corynebacterium equi*, *Jensenia canicruria*, and the soil strain of *Nocardia*. The results for the mycobacteria are in general agreement with those of Cerbon & Bojalil (1961), who found that *Mycobacterium smegmatis* and *M. phlei* were on the same branch of their dendrogram, the branch dividing at the 60 % level into two groups. It is of interest here that *C. equi* and *J. canicruria* appear in the mycobacteria group. Jensen (1934) placed *C. equi* in the genus *Mycobacterium* because of its acid-fastness, and Jensen (1952) considered that its existence showed that there was a close relationship between the genera *Corynebacterium* and *Mycobacterium*. The strain examined here showed no trace of acid-fastness. The genus *Jensenia* was proposed by Bisset & Moore (1949) to include the unicellular soil diphtheroids, but *J. canicruria*, described by these authors in 1950, has never gained much support as a specific entity, being identified as a nocardia by some authors and as a mycobacterium by others (see Adams & McClung, 1960; Gordon & Mihm, 1961; Kwapinski, 1964; Jones & Bradley, 1964). Sneath & Cowan (1958) found it to be in the mycobacterium group of their numerical taxonomy.

Corynebacterium rathayi, *C. betae* and *C. michiganense* appear to be more distinct from most of the animal parasitic corynebacteria, particularly those of the *C. diphtheriae* group, than do *C. fascians* and *C. fimi*, and this distinction is similar to that found with the cell-wall composition patterns of these organisms. Da Silva & Holt (1965) have compared some plant pathogens with *C. diphtheriae* and they concluded that the plant pathogens were sufficiently distinct from *C. diphtheriae* to warrant their exclusion from the genus *Corynebacterium*. They suggested that *C. fascians*, being so different from all the other coryneforms they examined, should be excluded from the family Corynebacteriaceae, a proposal the present author cannot agree with. Of the plant pathogens, *C. fascians* appears from the numerical taxonomy presented here, to be the one most closely related to the animal strains, a relationship that is also suggested by the cell-wall studies of Cummins (1962*b*).

The position of two of the strains from animal sources cannot be readily explained. *Corynebacterium segmentosum*, a species no longer recognised by *Bergey's Manual* (1957), is from the human respiratory tract, and *C. bovis* is commonly found in cow's milk, yet both fall into the Arthrobacter-plant pathogens group at the 51 % level of the dendrogram.

The large group at the 51 % level contains almost all the strains with the cell-wall composition which Cummins & Harris (1958) and Cummins (1962*b*) found to be typical of the true corynebacteria, the mycobacteria and the nocardias, and their presence in this group could further indicate a close relationship between these three genera.

In the distinction between and the separation of the genera *Corynebacterium* and *Mycobacterium*, the character of acid-fastness has always been a major factor, the corynebacteria being described as non-acid-fast and the mycobacteria as acid-fast. However, 'acid-fast' does not seem to be a well-defined term, and although its meaning is clear in medical laboratory procedure referring to *M. tuberculosis*, it is not always certain that the same exact meaning is used for other species of this genus and other genera. *Bergey's Manual* (1957) uses such descriptions as 'weakly acid-fast', 'acid-fast when grown under the proper conditions' and 'not as strongly acid-fast as . . .', which confirm the idea that the criterion of acid-fastness is not a sound taxonomic one. This is further supported by the results of the acid-fast staining reported here, in which the 'gravis', 'mitis' and p.w.8 strains of *C. diphtheriae*, and *C. ulcerans* were resistant to decolorization by 5 % sulphuric acid when grown in the presence of 2 % glycerol, and on this medium the oral nocardia was as acid-fast as the four mycobacteria.

The results of this present work, taken with the results of Kwapinski (1956, 1964), Cummins & Harris (1958) and Cummins (1962*b*), and the morphology of these organisms, lead the present author to support the suggestion, made by Cummins (1962*a*) on the basis of cell wall composition, that the genera *Corynebacterium*, *Mycobacterium* and *Nocardia* could be merged to form one genus. Even if this is not generally accepted, there is no reason to continue the separation of the genus *Corynebacterium* from the genera *Mycobacterium* and *Nocardia* into different orders mainly on the basis of acid-fastness.

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