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Nucleic Acid Hybridization Studies on *Microbacterium*, *Curtobacterium*, *Agromyces* and Related Taxa

By HANNELORE DÖPFER,[1] ERKO STACKEBRANDT[2]* AND FRANZ FIEDLER[1]

[1]*Lehrstuhl für Mikrobiologie der Universität München, Maria-Ward-Strasse 1a, 8000 München 19, Federal Republic of Germany*

[2] *Lehrstuhl für Mikrobiologie der Technischen Universität München, Arcisstrasse 21, 8000 München 2, Federal Republic of Germany*

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Thirty strains of *Agromyces*, *Arthrobacter*, *Curtobacterium*, *Brevibacterium*, *Corynebacterium* and *Microbacterium*, exhibiting the rare peptidoglycan of group B, were subjected to extensive nucleic acid hybridization studies. The DNA homology values indicate that *Corynebacterium insidiosum* DSM 20157 is genetically identical with *Corynebacterium michiganense* DSM 20134. *Corynebacterium sepedonicum* NCPPB 378 and *Corynebacterium nebraskense* DSM 20400 are closely related to *Corynebacterium michiganense* DSM 20134. *Corynebacterium betae* DSM 20141, *Corynebacterium oortii* ATCC 25283 and *Corynebacterium poinsettiae* ATCC 9682 are genetically identical with *Corynebacterium flaccumfaciens* DSM 20129. In addition, *Curtobacterium citreum* ATCC 15828, *Curtobacterium luteum* ATCC 15830 and *Curtobacterium pusillum* ATCC 19096 share a high degree of relatedness to *Corynebacterium flaccumfaciens* DSM 20129. All other described species are more distantly related to each other. DNA–rRNA cistron similarity studies reveal that all coryneforms with a peptidoglycan group B are members of one homogeneous cluster for which the rank of a genus is suggested.

INTRODUCTION

The determination of the peptidoglycan type is one of the most reliable methods in the classification of Gram-positive bacteria (Schleifer & Kandler, 1972). Especially among the actinomycetes and coryneforms, the peptidoglycan type allows distinction to be made between groups of organisms which can not be unambiguously classified on the basis of morphology and other simple characteristics alone (Fiedler *et al.*, 1970; Schleifer, 1970; Schleifer & Kandler, 1972; Fiedler & Kandler, 1973; Keddie & Cure, 1977). Most of the groups defined by the peptidoglycan type show, in addition, a high similarity in other characteristics of taxonomic value, e.g. the isoprenoid composition (Collins *et al.*, 1979, 1980; Collins & Jones, 1981), the lipid composition (Minnikin *et al.*, 1978; Collins & Jones, 1980); and the cytochrome pattern (Faller, 1980). Moreover, the phylogenetic analysis of coryneform bacteria, using the 16S ribosomal RNA as a phylogenetic marker, has demonstrated a high degree of correlation between the grouping of organisms according to their natural relationship and the distribution of chemotaxonomic markers, defining these groups phenotypically (Stackebrandt *et al.*, 1980; Stackebrandt & Woese, 1981).

Most peptidoglycan types found within the eubacteria belong to the peptidoglycan group A (Schleifer & Kandler, 1972), defined by a cross-linkage extending from the ω-amino group of the diamino acid in position 3 of the peptide subunit to the carboxyl group of the C-terminal

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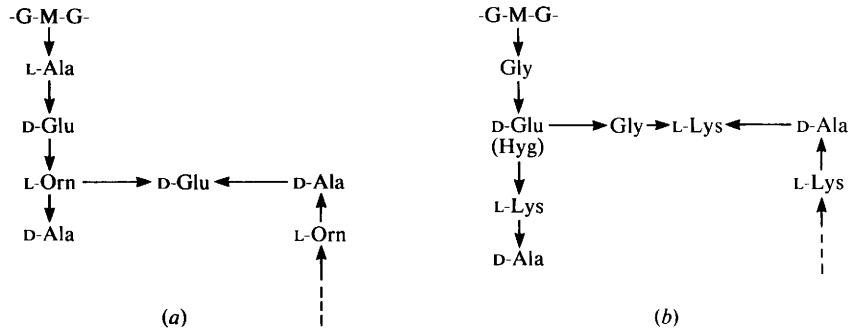

Fig. 1. Fragments of the primary structures of peptidoglycans of (*a*) group A (represented by *Cellulomonas cellasea*) and (*b*) group B (*Microbacterium lacticum*), according to Schleifer & Kandler (1972).

D-alanine in position 4 of an adjacent subunit (Fig. 1*a*). Only a small number of Gram-positive bacteria contain peptidoglycan types of group B, characterized by a cross-linkage between the α-carboxyl group of D-glutamic acid in position 2 of the peptide subunit and the C-terminal D-alanine of an adjacent subunit (Fig. 1*b*). Organisms containing this rare peptidoglycan of group B are found in coryneforms of the genera *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Microbacterium* (Fiedler *et al.*, 1970), in *Agromyces* (Fiedler & Kandler, 1973) and in *Curtobacterium* (Yamada & Komagata, 1972). It is also found in a few, mainly anaerobic organisms, namely *Clostridium barkeri* (Kandler & Andreesen, personal communication), *Acetobacterium woodii* (Kandler & Schoberth, 1979) and *Eubacterium limosum*, as well as in the aerobic organism *Erysipelothrix rhusiopathiae* (Schleifer & Kandler, 1972).

Clostridium barkeri, *Eubacterium limosum* and *Acetobacterium woodii*, which exhibit a low DNA G + C content ranging between 39 and 47 mol %, were recently found to be specifically related on the basis of oligonucleotide cataloguing of their 16S ribosomal RNA (Tanner *et al.*, 1981). Using the same method, the coryneform species *Microbacterium lacticum*, *Corynebacterium betae* and '*Corynebacterium mediolanum*' [names in inverted commas are not on the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) and have not been validly published since January 1, 1980] were found to form a cluster distinct from genera harbouring strains with peptidoglycan of group A, namely *Arthrobacter*, *Brevibacterium*, *Cellulomonas* and *Corynebacterium*, which themselves are clearly separated from each other phylogenetically.

It is now generally accepted that the genus *Corynebacterium*, represented by its type species *C. diphtheriae* in the phylogenetic studies, should be restricted to the human and animal corynebacteria and related saprophytic strains (Barksdale, 1970; Schleifer & Kandler, 1972; Jones, 1975; Collins *et al.*, 1982) with the plant pathogenic corynebacteria being excluded from this genus. Similarly, *Cellulomonas* (Stackebrandt *et al.*, 1980, 1982), *Brevibacterium* (Fiedler *et al.*, 1981; Yamada & Komagata, 1972; Jones, 1975, 1978; Collins *et al.*, 1980) and *Arthrobacter* (Stackebrandt & Fiedler, 1979; Stackebrandt *et al.*, 1980) are phylogenetically well defined genera.

The taxonomic status of the coryneforms containing a peptidoglycan of group B, on the other hand, is still unsettled. These organisms were not found to form a closely related cluster in numerical taxonomic studies (Da Silva & Holt, 1965; Davis & Newton, 1969; Davis *et al.*, 1969; Stuart & Pease, 1972; Jones, 1975; Seiler *et al.*, 1977) or comparative physiological studies (Yamada & Komagata, 1970, 1972; Keddie & Cure, 1977), but they clustered together with organisms containing peptidoglycan of group A. However, most of these studies were performed on a few representatives of each taxon only.

To determine the phylogenetic relationship of peptidoglycan group B-containing coryneform bacteria, 30 strains from 26 species were included in DNA–DNA homology and DNA–rRNA cistron similarity studies for the recognition of degrees of relatedness of closely related and of more remotely related species, respectively. The advantages and the limitations of the former method have been pointed out by Steigerwalt *et al.* (1976) and Stackebrandt *et al.* (1981), those of the latter method by De Smedt & De Ley (1977) and Stackebrandt *et al.* (1981).

METHODS

Bacteria used and culture conditions. The strains investigated, their sources and their peptidoglycan types are listed in Table 1.

Most of the organisms were cultivated in a tryptone (Oxoid)/yeast extract (Oxoid)/glucose/NaCl broth (1:0·5:0·5:0·5%, w/v), pH 7·4 *Agromyces ramosus* was grown in CASO (Merck) broth (3·9%, w/v), supplemented with yeast extract (0·3%, w/v), pH 7·4 and *Arthrobacter terregens* in 'terregens assay medium' (Reich & Hanks, 1964), supplemented with acetylacetone as 'terregens factor' (0·01%, w/v; Morrison *et al.*, 1965). For labelling RNA, cells were grown in a tryptone/yeast extract/glucose/NaCl broth (0·5:0·5:0·2:0·2%, w/v), or in CASO broth (3%, w/v), without additional yeast extract. All organisms were cultivated aerobically by shaking; large batches (15 l) were vigorously aerated through a submerged sintered glass filter. *Arthrobacter terregens, Brevibacterium imperiale, Corynebacterium insidiosum, Corynebacterium nebraskense* and *Corynebacterium sepedonicum* were grown at 23 °C, and all of the other strains at 30 °C.

Isolation of DNA. Unlabelled and labelled DNAs were purified according to Marmur (1961), modified as described by Stackebrandt & Kandler (1979). In some cases the purity of the DNA was improved by phenol treatment (Kirby, 1957): phenol, saturated with 1× SSC (0·15 mol NaCl and 0·015 mol tri-sodium citrate l^{-1}, pH 7·0) was added to an equal volume of the DNA solution and the mixture slowly shaken for 1 min at room temperature. After separating the phases by centrifugation, phenol remaining in solution in the DNA-containing phase was removed by shaking with chloroform (1 vol.).

For labelling DNA, [^{3}H]thymidine (New England Nuclear, 21·8 Ci $mmol^{-1}$; 807 GBq $mmol^{-1}$) was added to the broth before inoculation. At the beginning of the experiments organisms were labelled in 300 ml broth containing 1 mCi of the labelled compound. To increase the specific activity of the DNA the volume was later reduced to 50 ml containing 0·7 mCi of the labelled compound. Furthermore, in some strains the specific activity was improved by replacing [^{3}H]thymidine by [^{3}H]adenine (New England Nuclear, 14·1 Ci $mmol^{-1}$; 522 GBq $mmol^{-1}$). The specific activities ranged between 3×10^3 and $3{\cdot}5 \times 10^4$ c.p.m. $(\mu g\ DNA)^{-1}$.

Isolation of ribosomal 23S RNA. Cells were cultivated in 100 ml broth containing 1 mCi [^{3}H]uracil (New England Nuclear, 40·0 Ci $mmol^{-1}$; 1·48 TBq $mmol^{-1}$. To obtain undegraded RNA, cells were harvested in the exponential phase of growth. The isolation procedure was performed according to Stackebrandt *et al.* (1981). The specific activities ranged between $6{\cdot}7 \times 10^4$ and $1{\cdot}1 \times 10^5$ c.p.m. $(\mu g\ 23S\ rRNA)^{-1}$.

DNA–DNA hybridization. The procedures and modifications were described by Stackebrandt & Kandler (1979). Hybridization was carried out in Eppendorf vials containing 15 to 20 μg of unlabelled, filter-bound DNA and, depending on the specific activity, 0·1, 0·5 or 1·0 μg [^{3}H]DNA dissolved in 0·2 ml 3× SSC, adjusted to 30% formamide, at 60 °C for 30 h. Reaction conditions correspond to 25 °C below the T_m of the DNA (referring to an average DNA G + C content of 74 mol %), according to Marmur & Doty (1962). The average binding efficiency in the homologous system was 35%. All hybridization values were normalized to the homologous values designated as 100%.

DNA–rRNA hybridization. For DNA–rRNA hybridization and the determination of the melting points of the DNA–rRNA heteroduplices the procedures of De Ley & De Smedt (1975) were slightly modified. After shearing [^{3}H]rRNA by passing it through a French pressure cell (20000 lbf in^{-2}; 138 MPa), 0·2 ml heat-denaturated ^{3}H-labelled 23S rRNA solution (1 μg ml^{-1}) was added to each filter, carrying 15–20 μg DNA. The hybridization buffer was 5× SSC adjusted to 25% formamide. After 12 h incubation at 60 °C, the [^{3}H]rRNA solution was sucked off and the filters were subsequently incubated at 60 °C for 10 min with 1 ml hybridization buffer to remove non-specifically bound [^{3}H]rRNA. The filters were then washed in cold 5× SSC, followed by RNAase treatment for 45 min at 37 °C in 1 ml 2× SSC, containing 0·25 μg RNAase A (Boehringer) ml^{-1} and 5 U RNAase T_1 (Calbiochem) ml^{-1}. After washing with cold 2× SSC, filters were dried at 60 °C *in vacuo* and the bound radioactivity determined. The percentage of rRNA binding was calculated according to De Smedt & De Ley (1977). After determination of the radioactivity, filters were washed with toluene, dried and then placed in a vial containing 1 ml 1·5× SSC adjusted to 25% formamide. The thermal stability of the heteroduplices was determined between 50 °C and 90 °C after De Ley & De Smedt (1975).

Determination of the G + c mol % of DNA. The guanine (G) plus cytosine (C) content of the DNA was calculated from its thermal denaturation temperature (Mandel & Marmur, 1968). The melting profiles were determined in a Zeiss spectrophotometer (M4QIII).

Table 1. *Source, DNA G+C content and peptidoglycan variation and type of the organisms investigated*

Strain and source†	DNA G+C content	Peptidoglycan‡ Variation	Type
Microbacterium lacticum DSM 20427 (ATCC 8180)	74·9	B1α	[L-Lys]-D-Glu-Gly-L-Lys (Hyg)
'*Corynebacterium laevaniformans*' DSM 20140 (ATCC 15953)	73·7		
Brevibacterium imperiale ATCC 8365	75·4	B1β	[L-Hsr]-D-Glu-Gly_2-L-Lys (Hyg)
*Curtobacterium citreum** ATCC 15828	75·2	B2β	[L-Hsr]-D-Glu-D-Orn
*Curtobacterium luteum** ATCC 15830	74·9		
*Curtobacterium pusillum** ATCC 19096	74·2		
Corynebacterium betae DSM 20141 (ATCC 13437)	73·7		
Corynebacterium flaccumfaciens DSM 20129 (ATCC 6887)	72·2		
Corynebacterium flaccumfaciens var. *aurantiacum* DSM 20135 (ATCC 12813)	72·2		
Corynebacterium oortii ATCC 25283	72·2		
Corynebacterium poinsettiae ATCC 9682	72·5		
Arthrobacter terregens ATCC 13345	75·6		[L-Hsr]-D-Glu-Gly-D-Orn (Hyg)
'*Brevibacterium insectiphilum*' Bhat 146	72·5		
*Curtobacterium albidum** CCM 2296	72·0		
*Curtobacterium saperdae** DSM 20169 (ATCC 19272)	72·5		
*Curtobacterium testaceum** DSM 20166 (ATCC 15829)	72·7		
'*Corynebacterium barkeri*' DSM 20145 (ATCC 15954)	74·4		
Coryneform organism DSM 20143	75·6		
'*Microbacterium liquefaciens*' Robinson 15	72·5		
Corynebacterium bovis ATCC 13722	73·7		[L-Hsr]-D-Glu-Gly-D-DAB
'*Brevibacterium helvolum*' ATCC 13715	72·2	B2γ	[L-DAB]-D-Glu-D-DAB
'*Corynebacterium aquaticum*' DSM 20146 (ATCC 14665)	73·2		
Corynebacterium insidiosum DSM 20157 (ATCC 10253)	76·1		
'*Corynebacterium mediolanum*' DSM 20152 (ATCC 14004)	74·4		
Corynebacterium michiganense DSM 20134 (ATCC 10202)	74·2		
Corynebacterium michiganense ATCC 4410	–		
Corynebacterium michiganense ATCC 7429	–		
Corynebacterium nebraskense DSM 20400	76·6		
Corynebacterium sepedonicum NCPPB 378	74·9		
Agromyces ramosus DSM 43045 (ATCC 25173)	76·7		
Cellulomonas cellasea DSM 20118 (ATCC 487)	75·0	A4β	L-Orn-D-Glu
Cellulomonas uda DSM 20107 (ATCC 491)	72·0		
Lactobacillus curvatus DSM 20019 (ATCC 25601)	42·9	A1α	L-Lys-D-Asp
Staphylococcus aureus ATCC 12600	31·2	A3α	L-Lys-Gly_{5-6}

–, Not investigated.

* These organisms are also listed as *Brevibacterium* in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) as objective synonyms.

† Abbreviations: ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; CCM, Czechoslovak Collection of Micro-organisms, Brno, Czechoslovakia; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, F.R.G.; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.; Bhat, J. V. Bhat, Indian Institute of Science, Bangalore, India; Robinson, K. Robinson, North of Scotland School of Agriculture, Aberdeen, U.K.; Seiler, H. Seiler, Technical University, Munich-Weihenstephan, F.R.G.

‡ Peptidoglycan variation and type are abbreviated as proposed by Schleifer & Kandler (1972). Lys, lysine; Gly, glycine; Hsr, homoserine; Orn, ornithine; Glu, glutamic acid; Hyg, hydroxyglutamic acid; DAB, diaminobutyric acid; Asp, aspartic acid. Amino acids in brackets refer to position 3; D-Glu refers to position 2 of the peptide subunit; the following amino acids form the interpeptide bridge. D-Glu can be partially replaced by *threo*-3-hydroxyglutamic acid (this information is omitted in the text and the following tables).

RESULTS

G+C mol % of DNA

The G+C mol% of DNA of all coryneforms investigated ranged between 72·0 and 76·1 (Table 1).

DNA–DNA hybridization

The homology values obtained are listed in Tables 2 to 5. Strains listed in Tables 2 to 4 are arranged by their peptidoglycan type. Organisms of the [L-Hsr]-D-Glu-D-Orn peptidoglycan type (B2β) share moderate to high DNA homology (44–104%) (Table 2). The highest homology values are found between *Corynebacterium betae* DSM 20141, *C. flaccumfaciens* DSM 20129, *C. flaccumfaciens* var. *aurantiacum* DSM 20135, *C. oortii* ATCC 25283 and *C. poinsettiae* ATCC 9682 (70–104%). *Curtobacterium citreum* ATCC 15828, *Curtobacterium luteum* ATCC 15830 and *Curtobacterium pusillum* ATCC 19096 reveal a somewhat lower DNA homology among each other as well as to the other strains of this group (44–56%). DNA homology values obtained for organisms containing peptidoglycan of variation B2β of the [L-Hsr]-D-Glu-Gly-D-Orn-type range between 16 and 36% (Table 3), indicating that the strains investigated are not closely related to each other. The highest values within this group are found between *Curtobacterium albidum* CCM 2296, *Curtobacterium saperdae* DSM 20169 and the coryneform organism DSM 20143 (34–39%), as well as between '*Brevibacterium insectiphilum*' Bhat 146 and '*Microbacterium liquefaciens*' strain Robinson 15 (28–30%). Low values were found with reference [^{3}H]DNA from '*Corynebacterium barkeri*' DSM 20145, *Arthrobacter terregens* ATCC 13345 and *Curtobacterium testaceum* DSM 20166, respectively, and DNA from other strains of this group (18–24%).

Among the phytopathogenic bacteria containing the peptidoglycan type [L-DAB]-D-Glu-D-DAB (B2γ), *Corynebacterium insidiosum* DSM 20157, *C. michiganense* DSM 20134, *C. sepedonicum* NCPPB 378 and *C. nebraskense* DSM 20400 share the highest DNA homology (32–74%) (Table 4). All three strains of *C. michiganense* (DSM 20134, ATCC 4410, and ATCC 7429) share 93 to 100% homology (values are not shown in Table 4), and the pair *C. insidiosum* DSM 20157 and *C. michiganense* DSM 20134 also shows a close relationship (74%).

'*Corynebacterium aquaticum*' DSM 20146, '*C. mediolanum*' DSM 20152, *Agromyces ramosus* DSM 43045, and '*Brevibacterium helvolum*' ATCC 13715 are not specifically related to any other strain of this peptidoglycan cluster (14–27%).

Microbacterium lactum DSM 20427 and '*Corynebacterium laevaniformans*' DSM 20140, organisms showing the peptidoglycan variation [L-Lys]-D-Glu-Gly-L-Lys (B1 α-type) are moderately related to each other (44% homology) (Table 5).

Table 5 also shows homology values found among members of the different groups listed in Tables 2 to 4, together with those obtained with reference [^{3}H]DNA from *Brevibacterium imperiale* ATCC 8365 and *Corynebacterium bovis* ATCC 13722, which are the only representatives of groups defined by peptidoglycan of the variation B1β and B2β of the [L-Hsr]-D-Glu-Gly-D-DAB-type, respectively. No close homology is found between representatives of these groups. Moderately high values are found for groups represented by *Microbacterium lacticum* DSM 20427 (B1α), *Brevibacterium imperiale* ATCC 8365 (B1β), and *Arthrobacter terregens* ATCC 13345 (B2β), of the [L-Hsr]-D-Glu-Gly-D-Orn-type) (18–34%). In general the values are in the same range (10–20%) as those found for remotely related organisms showing the same peptidoglycan variation (8–25%, Tables 3 and 4).

Corynebacterium bovis ATCC 13722, which contains a unique peptidoglycan type, is more distantly related to the other organisms tested (8–16%). These values are only slightly higher than those values obtained between organisms containing peptidoglycan of group A and B (7–13%).

DNA–rRNA hybridization

To decide whether organisms sharing low DNA–DNA homology values are actually related at the species level, or whether they have to be regarded as members of different genera, DNA–rRNA hybridization experiments were performed. *Corynebacterium betae* DSM 20141, *Microbacterium lacticum* DSM 20427, *Agromyces ramosus* DSM 43045, and

Table 2. *Percentage DNA homologies among strains with the* [L-*Hsr*]-D-*Glu*-D-*Orn peptidoglycan type* (*variation B2β*)

	[^{3}H]DNA from strain:							
Filter-bound DNA from strain:	DSM 20141	DSM 20129	DSM 20135	ATCC 25283	ATCC 9682	ATCC 12828	ATCC 15830	ATCC 19096
Corynebacterium betae DSM 20141	100	73	80	74	80	51	56	51
C. flaccumfaciens DSM 20129	77	100	102	78	83	48	45	54
C. flaccumfaciens var. *aurantiacum* DSM 20135	73	104	100	82	99	48	51	49
C. oortii ATCC 25283	79	71	81	100	71	51	50	54
C. poinsettiae ATCC 9682	75	80	99	78	100	46	45	50
Curtobacterium citreum ATCC 15828	48	–	56	52	47	100	44	53
Curtobacterium luteum ATCC 15830	–	–	–	–	–	51	100	49
Curtobacterium pusillum ATCC 19096	50	–	54	50	51	52	48	100

–, Not investigated.

Table 3. *Percentage DNA homologies among strains with the* [L-*Hsr*]-D-*Glu-Gly*-D-*Orn peptidoglycan type* (*variation B2γ*)

	[^{3}H]DNA from strain:							
Filter-bound DNA from strain:	DSM 20145	CCM 2296	DSM 20169	Bhat 1461	Rob. 15	DSM 20143	ATCC 13345	DSM 20166
'*Corynebacterium barkeri*' DSM 20145	100	20	23	23	21	23	17	23
Curtobacterium albidum CCM 2296	–	100	37	–	–	36	–	–
Curtobacterium saperdae DSM 20169	–	34	100	–	–	39	–	–
'*Brevibacterium insectiphilum*' Bhat 146	22	21	25	100	28	32	18	21
'*Microbacterium liquefaciens*' Robinson 15	21	–	–	30	100	32	20	22
Coryneform organism DSM 20143	22	–	–	27	–	100	–	18
Arthrobacter terregens ATCC 13345	18	16	16	18	23	22	100	24
Curtobacterium testaceum DSM 20166	22	17	17	21	21	21	24	100

–, Not investigated.

Table 4. *Percentage DNA homologies among strains with the* [L-*DAB*]-D-*Glu*-D-*DAB peptidoglycan type* (*variation B2γ*)

	[^{3}H]DNA from strain:						
Filter-bound DNA from strain:	DSM 20157	DSM 20134	DSM 20400	DSM 20146	DSM 20152	DSM 43045	ATCC 13715
Corynebacterium insidiosum DSM 20157	100	73	48	18	16	17	17
Corynebacterium michiganense DSM 20134	74	100	32	17	14	18	12
Corynebacterium sepedonicum NCPPB 378	51	56	39	14	13	18	16
Corynebacterium nebraskense DSM 20400	45	46	100	15	13	15	12
'*Corynebacterium aquaticum*' DSM 20146	17	19	23	100	22	21	17
Corynebacterium mediolanum DSM 20152	20	16	19	22	100	27	21
Agromyces ramosus DSM 43045	19	18	19	25	25	100	21
'*Brevibacterium helvolum*' ATCC 13715	15	14	20	17	16	18	100

Corynebacterium bovis ATCC 13722 served as the source for ^{3}H-labelled 23S rRNA. The results are summarized in Table 6 in terms of percentage of rRNA binding, the midpoint of elution profiles of DNA–rRNA heteroduplices ($T_{m(e)}$) and the $\Delta T_{m(e)}$ values. The $T_{m(e)}$ values have proven more reliable for the recognition of relationships than did the amount of rRNA bound to filter-fixed DNA (De Ley & De Smedt, 1975; Mordarski *et al.*, 1980; Stackebrandt

Table 5. *Range of percentage DNA homologies between organisms of groups defined by different peptidoglycan types*

Filter-bound DNA from organisms with peptidoglycan type:		Representative species	[^{3}H]DNA from organisms with peptidoglycan type: (*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)
Of group B								
[L-Lys]-D-Glu-Gly-L-Lys	(*a*)	*M. lacticum*	100					
		'*C. laevaniformans*'	44					
[L-Hsr]-D-Glu-Gly_2-L-Lys	(*b*)	*B. imperiale*	29–34	100				
[L-Hsr]-D-Glu-D-Orn	(*c*)	*C. flaccumfaciens*	19–20	15–20	X*			
[L-Hsr]-D-Glu-Gly-D-Orn	(*d*)	*A. terregens*	18–33	19–25	10–20	X*		
[L-DAB]-D-Glu-D-DAB	(*e*)	*C. michiganense*	9–19	13–21	11–22	10–20	X*	
[L-Hsr]-D-Glu-Gly-D-DAB	(*f*)	*C. bovis*	8–13	12	11–13	11–15	10–16	100
Of group A								
L-Orn-D-Glu		*Cellulomonas uda*			7–11			
L-Orn-D-Glu		*Cell. cellasea*			8–13			

* Homology values found among members of the individual groups are listed in Tables 2 to 4.

et al., 1981; Kilpper-Bälz & Schleifer, 1981). The differences between the $T_{m(e)}$ values of the homologous and heterologous reaction ($\Delta T_{m(e)}$) spread over a rather narrow range of about 8 °C. Small $\Delta T_{m(e)}$ values ($\leqslant$4 °C) were found between heteroduplices formed with reference [^{3}H]rRNA from *M. lacticum* and DNA from '*C. laevaniformans*' and strains showing peptidoglycan variation B1β and B2β ([L-Hsr]-D-Glu-Gly-D-Orn-type), as well as with DNA from *Agromyces ramosus*. Similar small values were found with reference [^{3}H]rRNA from *C. betae* and DNA from other members showing the identical peptidoglycan type, as well as between reference [^{3}H]rRNA from *Agromyces ramosus* and DNA from *Arthrobacter terregens*. The highest $\Delta T_{m(e)}$ values among coryneforms with peptidoglycan of group B (6·5–8 °C) were found when DNA or [^{3}H]rRNA from *C. bovis* ATCC 13722 was involved in the formation of heteroduplices.

Organisms showing peptidoglycan of group A, which served as remotely related reference organisms, have generally higher $\Delta T_{m(e)}$ values (7·5–16·0 °C), depending on the degree of relatedness to the reference [^{3}H]rRNAs.

The degree of rRNA cistron similarity between *Microbacterium lacticum* DSM 20427 and *Agromyces ramosus* DSM 43045, respectively, and representatives of the groups defined by the various peptidoglycan types, is expressed by means of similarity maps (De Smedt & De Ley, 1977) in Figs 2 and 3.

DISCUSSION

DNA–DNA hybridization studies on coryneforms containing peptidoglycan of group B (Schleifer & Kandler, 1972) do not allow a decision upon whether all organisms investigated are closely related, because only a few strains share high DNA homologies. Taking 70% homology as the borderline for species differentiation (Steigerwalt *et al.*, 1976) some of the described species have to be regarded as strains of one species. *Corynebacterium michiganese* DSM 20134 and *C. insidiosum* DSM 20157 turned out to be genetically identical, a finding that supports earlier results deduced from numerical data (Dye & Kemp, 1977). In the latter study *C. sepedonicum* and *C. nebraskense*, as well as '*C. iranicum*' and '*C. tritici*', were also described as strains of *C. michiganense*. DNA homology values obtained for *C. sepedonicum* NCPPB 378, *C. nebraskense* DSM 20400 and *C. michiganense* DSM 20134 indicate a close relatedness, though not at the strain, but at the species level. DNA homology studies by Starr *et al.* (1975) revealed that *C. insidiosum* NCPPB 2144 and *C. sepedonicum* (strain Burkholder) showed 100% homology, while *C. michiganense* and *C.*

Table 6. *Properties of DNA–rRNA heteroduplices of ^{3}H-labelled 23S rRNA from Microbacterium lacticum DSM 20427, Corynebacterium betae DSM 20141, Agromyces ramosus DSM 43045 and Corynebacterium bovis ATCC 13722, and DNA from various coryneform organisms containing peptidoglycan of groups A and B*

Peptidoglycan type	No. in Figs 2–3	Filter-bound DNA from:	^{3}H-labelled 23S rRNA from: *M. lacticum* % *	*M. lacticum* $T_{m(e)}$	*M. lacticum* $\Delta T_{m(e)}$	*C. betae* % *	*C. betae* $T_{m(e)}$	*C. betae* $\Delta T_{m(e)}$	*A. ramosus* % *	*A. ramosus* $T_{m(e)}$	*A. ramosus* $\Delta T_{m(e)}$	*C. bovis* % *	*C. bovis* $T_{m(e)}$	*C. bovis* $\Delta T_{m(e)}$
Of group B														
[L-Lys]-D-Glu-Gly-L-Lys	1	*Microbacterium lacticum*	0·17	81·5	†	0·12	75·0	4·5	0·20	76·0	5·0	0·20	73·0	6·5
	2	*'Corynebacterium laevaniformans'*	0·06	77·5	4·0	0·09	74·0	5·5	0·08	75·5	5·5	–	–	–
[L-Hsr]-D-Glu-Gly_2-L-Lys	3	*Brevibacterium imperiale*	0·24	80·0	1·5	0·25	73·5	6·0	0·30	76·5	4·5	0·43	72·5	7·0
[L-Hsr]-D-Glu-D-Orn	4	*Corynebacterium betae*	0·19	76·5	5·0	0·44	79·5	†	0·27	76·5	4·5	0·35	73·5	6·0
	5	*Corynebacterium oortii*	–	–	–	0·16	78·0	1·5	–	–	–	–	–	–
	6	*Corynebacterium poinsettiae*	0·28	75·0	6·5	–	–	–	0·39	74·5	6·5	–	–	–
	7	*Curtobacterium citreum*	0·36	74·5	7·0	0·48	78·0	1·5	0·41	76·0	5·0	–	–	–
[L-Hsr]-D-Glu-Gly-D-Orn	8	*Arthrobacter terregens*	0·10	78·5	3·0	0·07	74·5	5·0	0·10	77·5	3·5	–	–	–
	9	*Curtobacterium albidum*	0·20	78·5	3·0	0·18	74·0	5·5	0·28	75·0	6·0	–	–	–
	10	*'Brevibacterium insectiphilum'*	0·25	77·5	4·0	–	–	–	–	–	–	0·43	73·5	6·0
	11	*Curtobacterium saperdae*	0·14	79·5	2·0	–	–	–	–	–	–	–	–	–
	12	*Curtobacterium testaceum*	0·32	77·5	4·0	0·30	74·5	5·0	0·39	76·0	5·0	–	–	–
[L-DAB]-DGlu-D-DAB	13	*Agromyces ramosus*	0·15	78·5	3·0	0·16	75·0	4·5	0·25	81·0	†	0·23	73·5	6·0
	14	*'Brevibacterium helvolum'*	0·18	76·5	5·0	0·26	74·0	5·5	0·22	76·0	5·0	0·34	75·0	4·5
	15	*Corynebacterium insidiosum*	0·21	76·5	5·0	0·28	74·0	5·5	0·30	76·0	5·0	–	–	–
	16	*Corynebacterium michiganense*	–	–	–	0·24	74·5	5·0	–	–	–	–	–	–
	17	*Corynebacterium nebraskense*	0·19	77·0	4·5	–	–	–	0·26	76·0	5·0	–	–	–
	18	*'Corynebacterium aquaticum'*	0·05	76·0	5·5	0·16	74·0	5·5	–	–	–	–	–	–
	19	*'Corynebacterium mediolanum'*	0·18	77·0	4·5	–	–	–	–	–	–	–	–	–
[L-Hsr]-D-Glu-Gly-D-DAB	20	*Corynebacterium bovis*	0·12	75·0	6·5	0·26	73·0	6·5	0·29	73·0	8·0	0·46	79·5	†
Of group A														
L-Orn-D-Glu	21	*Cellulomonas cellasea*	–	–	–	–	–	–	0·05	73·0	8·0	0·10	71·0	8·5
	22	*Cellulomonas uda*	0·30	74·0	7·5	–	–	–	–	–	–	–	–	–
L-Lys-D-Asp	23	*Lactobacillus curvatus*	0·09	67·0	14·5	–	–	–	0·10	67·0	14·0	0·11	68·0	11·5
L-Lys-Gly_{5-6}	24	*Staphylococcus aureus*	0·16	65·0	16·5	–	–	–	0·17	65·0	16·0	0·20	66·0	13·5

–, Not investigated.
* rRNA binding to 100 μg DNA.
† Homologous reaction.

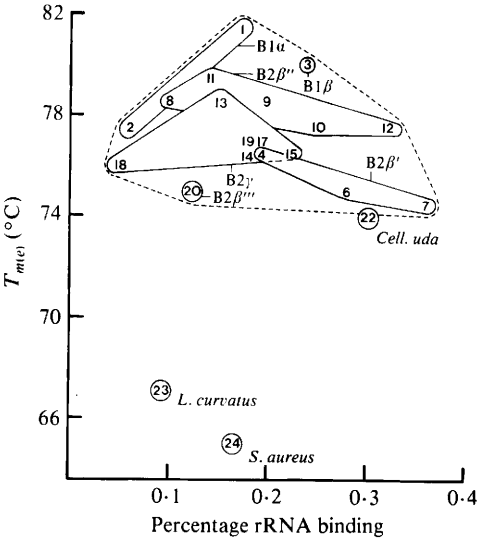

Fig. 2

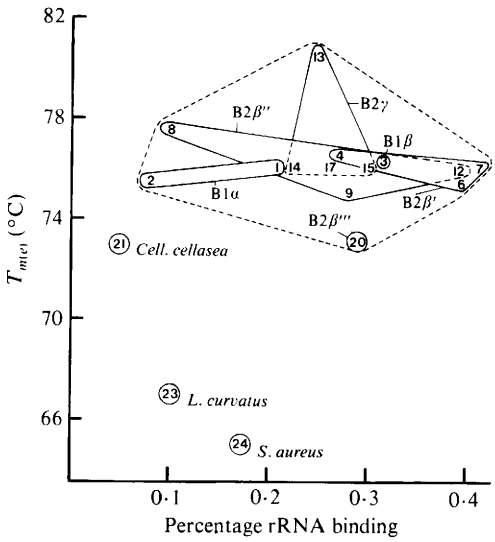

Fig. 3

Fig. 2. Similarity map of heteroduplices between ^{3}H-labelled 23S rRNA from *Microbacterium lacticum* DSM 20427 and DNA from various organisms containing peptidoglycan of group A and group B. Strains are designated by their sequence number (Table 6). B2β', [L-Hsr]-D-Glu-D-Orn; B2β'', [L-Hsr]-D-Glu-Gly-D-Orn; B2β''', [L-Hsr]-D-Glu-Gly-D-DAB. Organisms sharing the same peptidoglycan type are surrounded by continuous lines.

Fig. 3. Similarity map of heteroduplices between ^{3}H-labelled 23S rRNA from *Agromyces ramosus* DSM 43045 and DNA from various organisms containing peptidoglycan of group A and of group B. For additonal information see legend to Fig. 2.

tritici were found to be less related to *C. sepedonicum*. Despite these differences seen in the two investigations, (probably due to the selection of strains and methods), the high degree of relatedness is obvious and is further supported by the possession of an identical peptidoglycan type (Fiedler & Kandler, 1973; Schleifer & Kandler, 1972), as well as by high similarities in the composition of long-chain fatty acids and isoprenoid quinones (Minnikin *et al.*, 1978; Collins & Jones, 1980).

Another group of described species, which are actually related at the strain level, is formed by *C. flaccumfaciens* DSM 20129, *C. betae* DSM 20141, *C. oortii* ATCC 25283 and *C. poinsettiae* ATCC 9682. This accords with the results of a numerical phenetic analysis (Dye & Kemp, 1977), and with serological and cell wall analysis (Lelliott, 1966; Schleifer & Kandler, 1972; Keddie & Cure, 1977; Rosenthal & Cox, 1954). The other organisms belonging to the same group as defined by the peptidoglycan type, namely *Curtobacterium citreum* ATCC 15828, *Curtobacterium luteum* ATCC 15830 and *Curtobacterium pusillum* ATCC 19096, have to be regarded as closely related species. The close genetic relatedness between the latter three strains was also found recently by Suzuki *et al.* (1981). All these strains investigated share the same isoprenoid quinone composition and possess a characteristic polar lipid pattern (Collins *et al.*, 1980).

All the other coryneforms investigated that contained a peptidoglycan of group B, which were not found to be closely related to organisms around either *C. flaccumfaciens* or *C. michiganense*, have to be regarded as well-defined species. Interestingly, DNA homology values obtained between organisms containing peptidoglycan of subgroups B1 and B2 do not

clearly separate these organisms with respect to their peptidoglycan structures. The differences between these two peptidoglycan subgroups have not merely to be seen in the configuration of the diamino acids of the interpeptide bridges, but also in the enzymic 'outfit' responsible for the distinct linkage of these L- and D-amino acids.

Based on the high rRNA cistron similarity, as shown by the small range of $\Delta T_{m(e)}$ values, all coryneforms investigated possessing a peptidoglycan of group B are members of one rRNA cistron similarity cluster. Within this cluster those organisms sharing a high DNA–DNA homology also share a high rRNA cistron similarity. But what was already seen in the DNA–DNA hybridization studies (Table 5) is also found in the DNA–rRNA hybridization experiments: the genera *Microbacterium* and *Curtobacterium* cannot be distinguished phylogenetically. Those strains, classified as *Brevibacterium*, *Arthrobacter* and *Corynebacterium*, but belonging to the *Microbacterium–Curtobacterium* cluster are misclassified and have to be excluded from the genera to which they are allocated in the present classification.

The position of *Corynebacterium bovis* ATCC 13722 is not as clearcut as that of the other members of this cluster because DNA–rRNA heteroduplices using reference rRNA from *C. bovis* show the lowest $T_{m(e)}$ values. These are not significantly higher than those obtained with DNA from cellulomonads containing peptidoglycan of group A. The possession of a peptidoglycan of group B, together with the slightly higher rRNA cistron similarity, however, indicates that *C. bovis* ATCC 13722 is a true, though peripheral member of this cluster. *C. bovis* ATCC 13722 is a misclassified strain, in that it is not a representative of *Corynebacterium sensu stricto*. This strain is not related to *C. bovis* ATCC 7715, characterized by a *meso*-diaminopimelic acid-containing peptidoglycan, mycolic acid and the presence of arabinose and galactose in cell walls (Keddie & Cure, 1978). These features, which are characteristic for members of *Corynebacterium*, are definitely missing in *C. bovis* ATCC 13722 (F. Fiedler, unpublished).

Interestingly, *Agromyces ramosus* DSM 43045 is a member of the rRNA cistron similarity cluster. *Agromyces ramosus* shares several characters with *Actinomyces* and *Nocardia*, e.g. a substrate mycelium that fragments into coccoid and diphtheroid elements (Cross & Goodfellow, 1973). However, what was already seen in the close relatedness of *Oerskovia* and *Cellulomonas* (Stackebrandt *et al.*, 1982) is also true for *Agromyces*: the formation of a mycelium does not exclude a high genetic relationship to non-mycelium producing organisms.

It should be noted that the rather close relatedness observed between cellulomonads and coryneforms containing peptidoglycan of group B has already been found by the comparative analysis of their 16S rRNA (Stackebrandt *et al.*, 1980). The binary matching coefficients obtained for *C. betae* DSM 20141, '*C. mediolanum*' DSM 20152 and *M. lacticum* DSM 20427 ($S_{AB} = 0{\cdot}64$–$0{\cdot}73$) were only moderately higher than those found between these strains and representatives of *Cellulomonas* ($S_{AB} = 0{\cdot}53$–$0{\cdot}71$).

We are faced with the problem of deciding the taxonomic rank of this phylogenetically coherent cluster. The $\Delta T_{m(e)}$ values of the DNA–rRNA heteroduplices cover a range of 8 °C, with an average $\Delta T_{m(e)}$ of 6 °C (5 °C excluding *C. bovis* ATCC 13722). De Ley and his coworkers describe Gram-negative genera by $\Delta T_{m(e)}$ values in the range of about 5 °C (De Smedt & De Ley, 1977; Gillis & De Ley, 1980). Similar values were found for streptomycetes (6·3 °C), *Streptosporangium roseum* and relatives (7·6 °C), *Actinoplanes philippinensis* and relatives (5·3 °C) (Stackebrandt *et al.*, 1981), as well as for *Staphylococcus* (5·3 °C) (Kilpper-Bälz & Schleifer, 1981).

Considering the results of the DNA–rRNA cistron similarity studies together with the separate grouping of three representatives of this cluster in phylogenetic studies (Stackebrandt *et al.*, 1980), we think that the coryneform bacteria having a peptidoglycan of group B constitute a single genus, for which an emended genus *Microbacterium* will be proposed. Differences in the menaquinone pattern (Collins *et al.*, 1979; Collins & Jones, 1981), cytochrome pattern (Faller, 1980), lipid composition (Minnikin *et al.*, 1978; Collins & Jones,

1980) and peptidoglycan types (Schleifer & Kandler, 1972), found among strains tested, do not in our opinion carry enough weight to decide upon a separation into different genera. These differences may be helpful in the characterization at the species or subspecies level.

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