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A numerical analysis of ribosomal RNA gene patterns for typing clinical isolates of *Corynebacterium* group D2

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SUMMARY

Restriction digest fragments of DNA from 46 clinical isolates identified as *Corynebacterium* group D2, were separated by electrophoresis, Southern blotted onto nylon membranes and hybridized to a ribosomal RNA gene probe. The resulting band patterns were subjected to unweighed pair-group cluster analysis. Representative strains from the main clusters were compared with similarly prepared band patterns from type strains of human *Corynebacterium* species.

The results indicate that strains identified as *Corynebacterium* group D2 represent a unique taxon and that computer-assisted analysis of rRNA gene restriction fragment polymorphism (ribotyping) could be a useful technique in epidemiological studies of these bacteria.

INTRODUCTION

The analysis of rRNA gene restriction fragment polymorphism (ribotyping) as a tool in typing medically important bacteria is finding increasing use in epidemiological investigations. The principal advantage of the technique is that it enables all bacterial species to be typed, since all contain genomic rDNA sequences which are conserved and all strains are typable. Therefore, where serological, bacteriophage or other forms of typing are impracticable, ribotyping offers an alternative [1–3].

Here, we describe a computer-assisted analysis of the rRNA gene fragment profiles from urinary and other isolates of corynebacteria. These strains are similar in culture characteristics and antibiotic resistance to *Corynebacterium jeikeium* but differ from that species in their ability to hydrolyse urea and inability to acidify glucose [4]. Using classical biochemical tests, the Center for Disease Control, Atlanta, USA, have designated this phenon as *Corynebacterium* group D2 (CD2) [5]. Subsequent chemotaxonomic investigations have confirmed their membership of the genus *Corynebacterium* [6].

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CD2 bacteria have been implicated in significant bacteriuria sometimes leading to alkaline-encrusted cystitis and the formation of struvite (ammonium magnesium phosphate) calculi in the urinary tract [4, 7–9]. CD2 have also been reported as a cause of endocarditis [10]. Colonization of the skin of hospitalized patients is the probable source of infection [11]. The clinical aspects of CD2 infection have been reviewed by Soriano and Fernández-Roblas [4, 12] and by Coyle and Lipsky [13] but there have as yet, been no attempts to type these bacteria.

In this study, the rRNA gene profiles of 46 hospital isolates were subjected to numerical analysis using the Dice coefficient [14]. The resulting dendrograms enabled the discriminatory potential of the method to be assessed and provided evidence that CD2 is a relatively homogeneous taxon and distinct from other human species of *Corynebacterium*.

MATERIALS AND METHODS

Bacterial cultures

The sources of clinical isolates of CD2 used in this study are listed in Table 1. In addition, ten strains representing eight species of *Corynebacterium* from the National Collection of Type Cultures, London, UK, were included for comparison. These were; *C. cystitidis* NCTC 11863^T, *C. pilosum* NCTC 11862^T, *C. pseudotuberculosis* NCTC 3450^T, *C. pseudodiphtheriticum* NCTC 11136^T, *C. renale* NCTC 7448^T, *C. jeikeium* strains NCTC 11913^T, A504/88 and A512/88, '*C. genitalium*' NCTC 11859 and '*C. ulcerans*' NCTC 7910.

Stock cultures were maintained in the freeze-dried state and cultured routinely on 5% horse blood nutrient agar, aerobically at 37 °C. For DNA sample preparation, strains were grown in brain heart infusion broth supplemented with 0·4% yeast extract, 0·2% Tween 80 and 5% horse serum incubated at 37 °C with aeration. Strains were routinely identified by biochemical tests as specified by Hollis and Weaver [5].

Analysis of ribosomal RNA gene restriction patterns

Genomic DNA was extracted from centrifuged cells as previously described [15]. Restriction digestion of DNA samples was carried out using *Hin*d III or *Eco*R I endonucleases (Northumbria Biologicals, UK; 1 U/1 μg DNA) according to the manufacturer's instructions. The electrophoresis of restriction fragments, vacuum blotting, preparation and hybridization of a biotin-labelled rDNA probe have been previously described [16]. The source of rRNA was *Corynebacterium* sp. group D2 strain NCTC 12011.

Computer analysis

Banding profiles from *Hin*d III and *Eco*R I digests were combined and the total number of loci where bands occurred were counted.

The presence or absence of bands in loci was scored + or − for each strain. The percentage similarity of strains was estimated using the Dice coefficient [14]. Cluster analysis was by unweighed pair-group average linkage (UPGMA) [17].

Table 1. *Sources of strains analysed by ribotyping*

Source	Clinical origin	No. of strains
Fundación Jiménez Díaz, Madrid, Spain	Urine	15
	Skin	3
	Drain	1
	Calculus	1
	Blood	1
Hospital la Fuenfría, Madrid, Spain	Skin	14
Hospital la Princesa, Madrid, Spain	Urine	1
Ambulatorio González Bueno, Madrid, Spain	Not known	1
Hospital Mutua de Tarrasa, Barcelona, Spain	Urine	2
Hospital Nuestra Sra de Aránzazu, San Sebastián, Spain	Urine	2
Université Catholique Louvain, Belgium	Peritoneal fluid	1
	Blood	1
Southmead Hospital, Bristol, UK	Blood	2
	Renal wound	1

RESULTS

The banding profiles of clinical strains show a remarkable degree of homogeneity in both *Hin*d III and *Eco*R I DNA digests which is reflected in the low number of 26 loci overall in which bands occur (Fig. 1) in contrast to the species comparison (Fig. 3) where there are 66 loci overall. Individual profiles show 5–7 bands in *Hin*d III digests and 8 or 9 bands in *Eco*R I digests. In the *Hin*d III blot, a DNA fragment of approximately 4·8 kb hybridized in all strains and in the *Eco*R I blot similarly conserved bands corresponding to fragments of approximately 6 and 3 kb occur. More heterogeneity can be observed in the higher (> 5 kb) than in the lower (< 5 kb) molecular-weight bands.

Figure 2 is a simplified dendrogram representing cluster analysis calculated from the percentage similarity values of clinical isolates. To enable the maximum number of strains to be grouped, clusters were defined as groups of strains with $> 87\%$ similarity.

Forty-four of the 46 strains are related $> 74\%$ similarity and within this group, 7 clusters of 2 or more strains could be defined as well as 4 single strains. Although no overall correlation between the distribution of strains in clusters and either antibiotic resistance pattern (data not given), or origin and date of isolation was observed, some localized correlation between clustering and source of strain can be detected. The deepest division revealed by the analysis, at the 75% similarity level, separates clusters A–C from D–G. In the former three clusters, 11 of 16 strains are urinary isolates and 3 of 16 from skin, whereas in the remaining clusters, 8 of 24 strains are urinary isolates and 12 of 24 strains are from skin. Since the majority of urinary isolates are from the Fundación Jiménez Díaz, Madrid and the majority of skin isolates are from the Hospital la Fuenfría, Madrid, some of these strains formed subclusters coincident with place and time. In cluster B, six strains isolated at the former hospital in 1983–4, show $> 95\%$ similarity whereas four strains in cluster D ($> 89\%$ similarity) and four strains in cluster E ($> 97\%$ similarity) from the same source, were isolated in the period 1984–7. Also in cluster

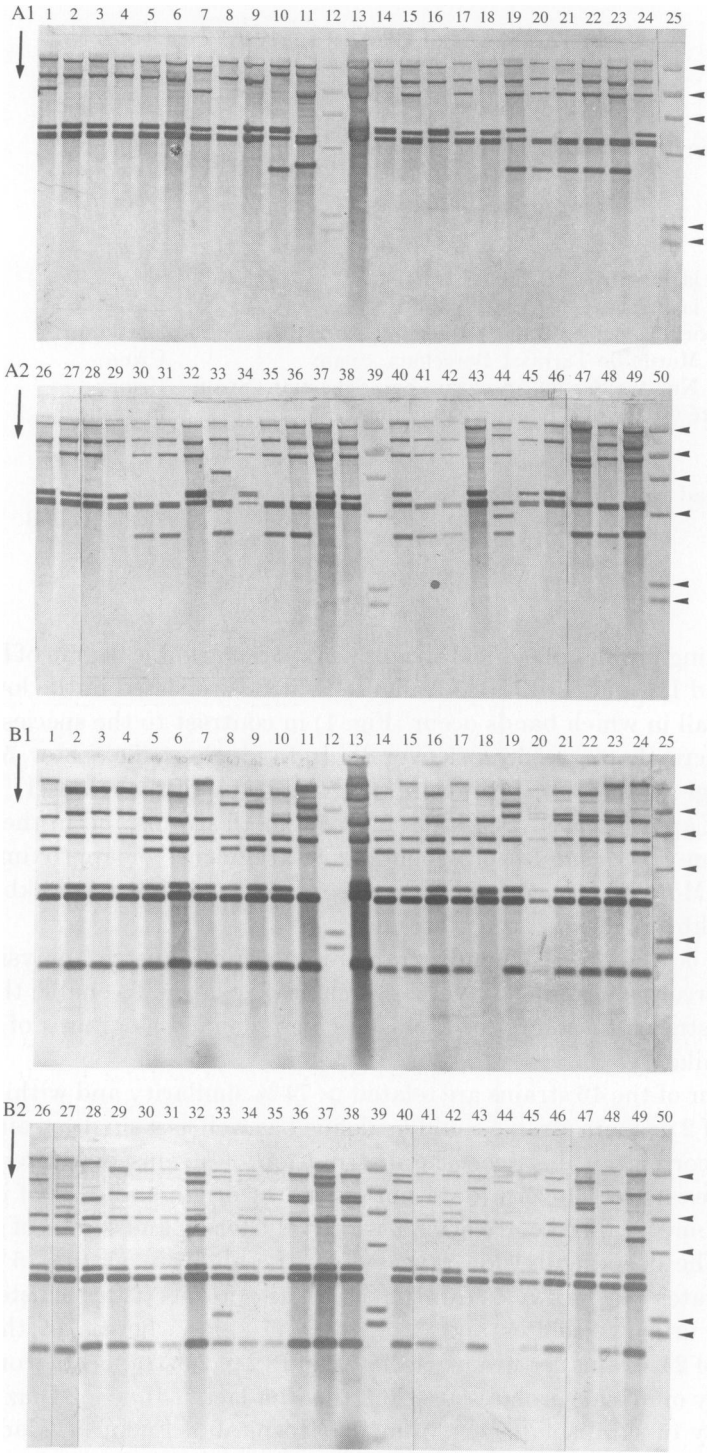

Fig. 1. Ribosomal RNA gene restriction fragment profiles of 46 strains of *Corynebacterium* group D2. Restriction endonucleases used were: A1, A2, *Hin*d III; B1, B2, *Eco*R I. Lanes 12, 25, 39, 50 molecular markers (*Hin*d III fragments of lambda phage DNA). From the top (in kb): 23·1, 9·4, 6·7, 4·4, 2·3, 2·0.

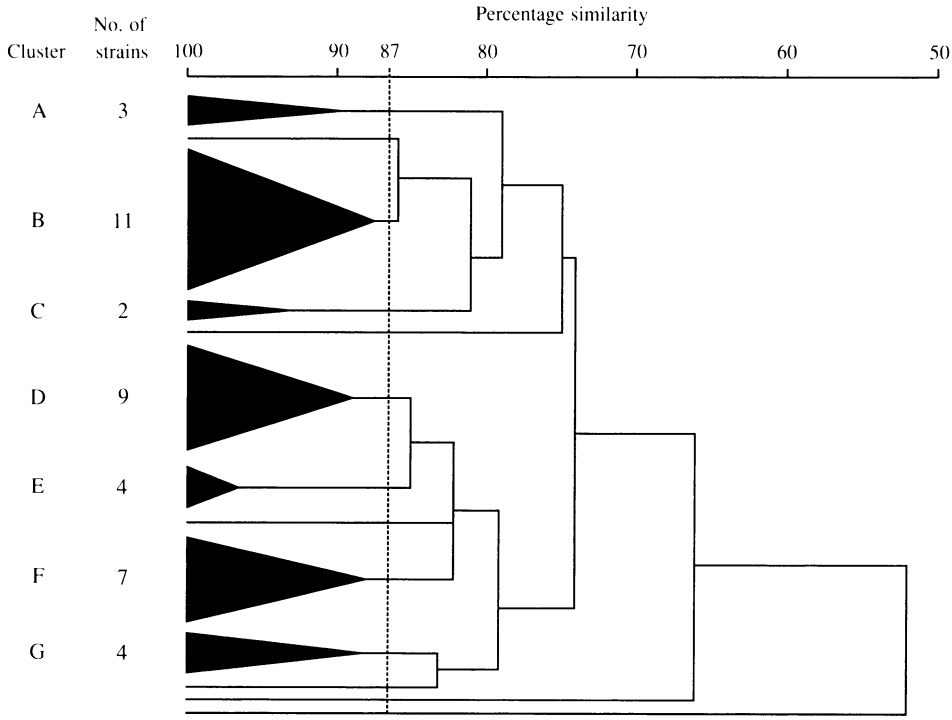

Fig. 2. Dendrogram showing cluster analysis of the rRNA gene restriction fragment profiles of 46 strains of *Corynebacterium* group D2 from Fig. 1. The percentage similarity values indicated on the horizontal axis were estimated using the Dice coefficient and UPGMA clustering. Ribotypes were defined as clusters of strains possessing > 87% similarity.

D, five strains from the Hospital la Fuenfría isolated in 1987 were 95% similar with four having identical profiles.

Figure 3 shows the banding profiles of six CD2 strains from clusters A, B, C, E, G and eight *Corynebacterium* species of human or animal origin of which *C. cystitidis*, *C. renale*, *C. pilosum*, '*C. ulcerans*', *C. pseudotuberculosis* and *C. pseudodiphtheriticum* are urealytic [18], and *C. jeikeium* and '*C. genitalium*' which are sometimes isolated from the genito-urinary tract but are non-urealytic [19, 20]. Figure 4 shows the dendrogram derived from these profiles. The CD2 strains 1–6 show > 74% inter-strain similarity in agreement with their position in Fig. 2 but only 40% similarity to any other species of *Corynebacterium*.

DISCUSSION

The purpose of this study was to evaluate the use of rRNA gene restriction profiles in identifying and typing urinary isolates of *Corynebacterium* group D2.

In the last decade, a number of studies have been carried out which indicate that this technique has considerable potential as a typing method [21]. The majority of studies have utilized a commercially available rRNA from *Escherichia*

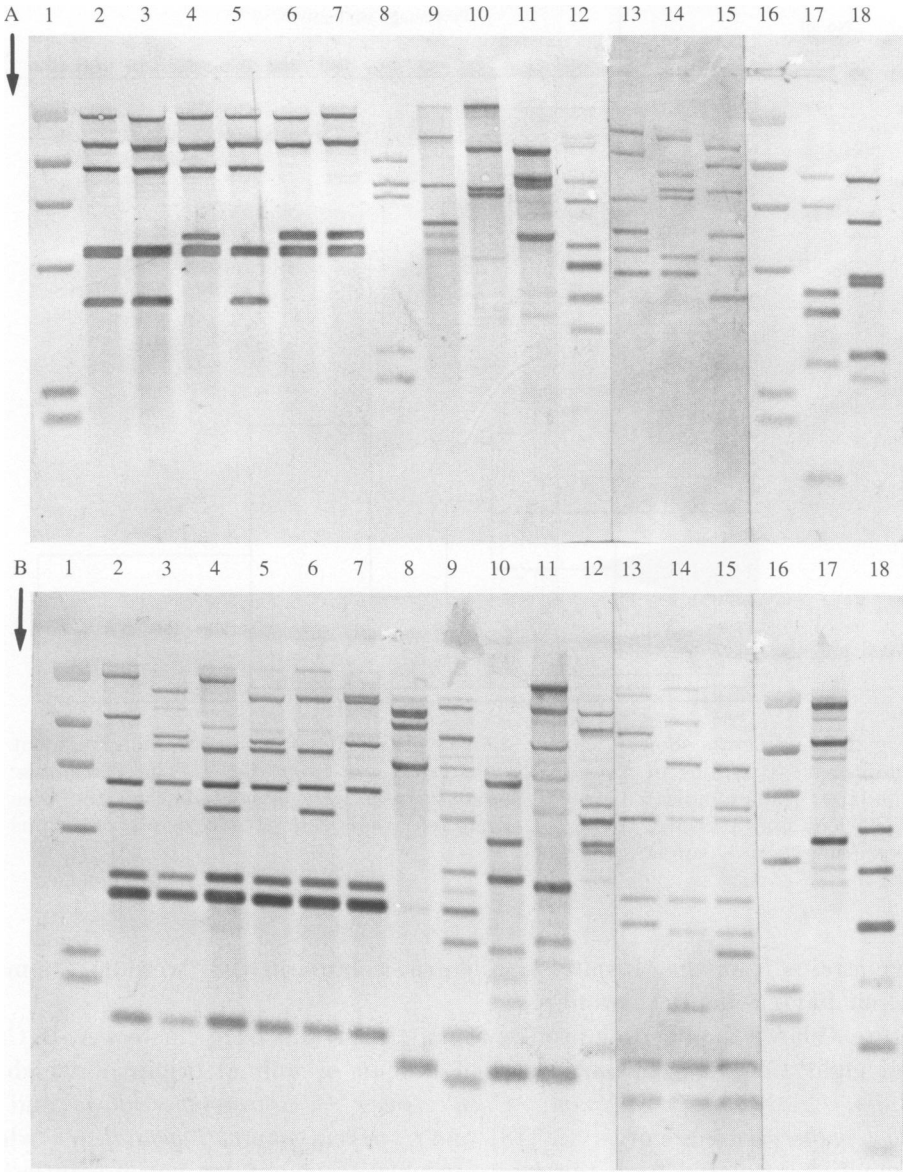

Fig. 3. Ribosomal RNA gene restriction fragment profiles of six strains of *Corynebacterium* group D2 and eight *Corynebacterium* species. Restriction endonucleases used were; (A) *Hin*d III; (B) *Eco*R I. Lanes 1, 16: marker fragments of *Hin*d III-digested λ-bacteriophage DNA (from the top in kb pairs): 23·1, 9·4, 6·7, 4·4, 2·3, 2·0. Lanes 2–7: *Corynebacterium* group D2 ribotypes A, B, D, B, F, E. Lane 8: *C. pilosum* NCTC 11862. Lane 9: *C. renale* NCTC 7448. Lane 10: '*C. ulcerans*' NCTC 9710. Lane 11: *C. pseudotuberculosis* NCTC 3450. Lane 12: '*C. genitalium*' NCTC 11859. Lanes 13–15: *C. jeikeium* A504/88, A512/88, NCTC 11913. Lane 17: *C. pseudodiphtheriticum* NCTC 11136. Lane 18: *C. cystitidis* NCTC 11863.

coli as a probe after labelling with ^{32}P. This has been referred to as a universal probe since sufficient of the sequence is conserved in different bacterial species for rRNA gene polymorphism to be detected [1, 2].

Our experience with *Corynebacterium* spp. using reverse transcriptase generated,

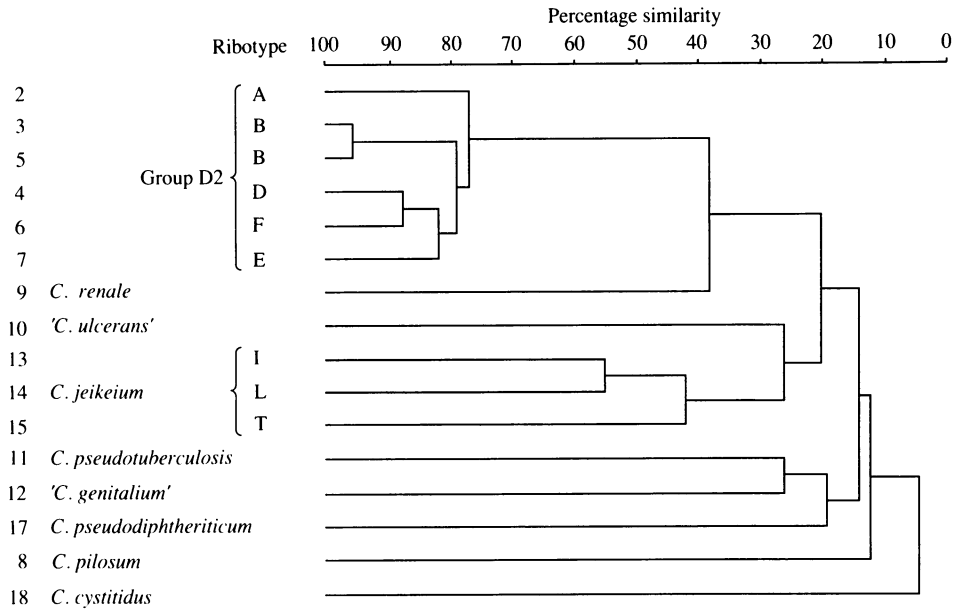

Fig. 4. Dendrogram showing the cluster analysis of rRNA gene restriction fragment profiles of *Corynebacterium* group D2 and eight *Corynebacterium* species. Numbers in the left-hand vertical column refer to the strain numbers listed in Fig. 3.

biotin-labelled probes, led us to use a template rRNA from the taxon being studied since this resulted in a much stronger signal than when *E. coli* rRNA was used (unpublished observations). Since several milligrams of rRNA can easily be prepared from a strain in a single extraction, sufficient rRNA can be made to supply several hundred probes. This also eliminates the need for cloned rRNA operons to be used as probes. The main advantage of biotin over ^{32}P as a reporter molecule is that, in addition to safety and half-life considerations, better resolution of the hybridization bands can be achieved enabling doublets to be detected.

Because urease activity appears to play a role in the pathogenesis of CD2 [4], it was thought that a close relationship might be found between CD2 and a named urealytic species of *Corynebacterium* inhabiting man or animals. The indication from ribotyping, is that this association is not evident, neither is there a close relationship with *C. jeikeium*, another multi-resistant species. It has also been suggested that CD2 may be a nitrate negative variant of *C. pseudodiphtheriticum* [5] but this is not supported by the present study.

There is a remarkable homogeneity in the profiles obtained for the 46 clinical isolates despite their having been acquired from a variety of sources, all but 2 were related by $> 74\%$ similarity and the majority could be assigned to 1 of 8 clusters at the $> 87\%$ similarity level. The two strains which were the exception, showed similarities of $< 66\%$ but $> 52\%$ to all other strains, which is nevertheless, a closer relationship than the inter-species relationships suggested in the dendrogram in Fig. 4. It is therefore probable that these strains have not been misidentified, but represent members of as yet undefined clusters.

Although there appeared to be some discrimination between urinary and skin

isolates, this may simply reflect the fact that most of the urinary isolates were collected in one hospital and most of the skin isolates in another. Therefore, neither clinical source or place of origin could be directly correlated with any cluster of strains. It is possible that future studies to ribotype urinary and skin isolates from the same patient and from cases where inter-patient contact is known, could resolve the question of strain transfer.

One restriction fragment band which hybridized to the rDNA probe on the *Hind* III blot and a further two bands on the *Eco*R I blot were observed to be present in all the strains and therefore represent highly conserved sequences in the genomes of strains in the CD2 taxon. These may be characteristic of a well delineated species as, in the case presented here, comparison with other corynebacteria (Fig. 3) did not reveal this combination of bands in any other species. It may therefore be of taxonomic value in the definition of a species to identify such highly conserved sequences.

Pitcher and co-workers [16] noted a close similarity (> 80%) between eight strains of *C. jeikeium* isolated in a single hospital unit, suggesting that those isolates could represent the cross-infection of patients with a single strain. However, the percentage similarity value for all 26 of the strains analysed, including reference strains was only 30%, indicating considerable rRNA gene polymorphism in this species. Three *C. jeikeium* strains were included in the present study. However, the interstrain similarity between them (40%), was less than that for all strains of CD2 suggesting that CD2 is a more homogeneous taxon than *C. jeikeium*.

This finding is supported by Hindmarch and co-workers [22] in a pyrolysis–mass spectrometric analysis of *Corynebacterium* spp., CD2 strains could be accommodated in a single cluster whereas *C. jeikeium* strains were located in six clusters.

Fatty acid analysis [23], mycolic acid structure [6] API profiles [24, 25] and classical biochemical tests [5, 7, 13, 26] also support the conclusion that CD2 is a relatively homogeneous and unique taxon within the genus *Corynebacterium*. We propose that computer assisted ribotyping could become a very useful method for studying the epidemiology of CD2 and other bacterial taxa which do not lend themselves to other methods of strain differentiation and that the demonstration of a number of conserved restriction fragments in the range 1–10 kb, containing sequences homologous with those of the rRNA operon contributes to the taxonomic delineation of a species.

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