rRNA gene RFLP as an identification tool for Corynebacterium species

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The value of rRNA gene RFLP analysis (ribotyping) as a tool for Corynebacterium and Turicella species identification was evaluated. Seventy-four strains representing 26 different species or subspecies were analysed by BstEII, SmaI and Sphl ribotyping. Numerical analysis of the resulting rDNA banding patterns was performed by Dice coefficient correlation in order to establish a database for species identification. In general, most of the strains belonging to the same species clustered together. Interestingly, BstEII clustering of many species followed known phylogenetic lineages. This was not evident with the more heterogeneous SmaI and Sphl patterns. The SmaI patterns contained a 1800 bp band in the digests of all species studied with the exception of Corynebacterium urealyticum. Sphl digestion resulted in the most heterogeneous patterns. The information provided by all three enzymes was considered essential for the reliable linking of strains of unknown identity with defined species in the database. It is concluded that ribotyping provides an useful tool for screening and characterization of potentially new Corynebacterium species.

Keywords: Corynebacterium, rRNA gene restriction patterns, ribotyping, identification, taxonomy

INTRODUCTION

Within the group of coryneform bacteria (i.e. aerobic, asporogenous, non-partially acid-fast, irregular, Gram-positive rods), the genus Corynebacterium contains the largest number of defined species (40 in March 1998). Bergey’s Manual of Systematic Bacteriology (Collins & Cummins, 1986) lists only 16 valid Corynebacterium species; however, between 1987 and 1995, 12 new species were defined (Funke et al., 1997d), and between 1996 and March 1998, another 11 new species were defined. Most of the species described during 1986–1998 were isolated from human clinical samples. The identification of corynebacteria from clinical samples, together with the appearance of newly established species, is still causing confusion for laboratories not used to dealing with the characteristics of this diverse genus.

Since it is most likely that a plethora of further new Corynebacterium species will be described in the near future, it was considered appropriate to evaluate the application of rRNA gene restriction fragment patterns (ribotyping) as a means of identification of coryneforms. The concept of ribotyping was established in the 1980s (Grimont & Grimont, 1986) and it has been used mainly for tracing the possible clonality of epidemiologically associated isolates. However, it was originally described as a potential tool for taxonomy and it is now used more and more for species identification. Several studies have shown that ribotyping deals with a genetically constant feature, resulting in species-specific bands or typical banding patterns within a species. For Gram-positive microbes, it has been used for identification within the genera Enterococcus (Hall et al., 1992), Streptococcus (Rudney & Larson, 1993), Lactobacillus (Björkroth & Korkeala, 1996, 1997) and Leuconostoc–Weissella (Björkroth et al., 1998).

The systematic use of ribotyping within the genus Corynebacterium has never been studied before. This study set out to evaluate the potential of this technique for the identification of Corynebacterium species. Several restriction enzymes were evaluated in order to establish a revealing database aiding the identification of these bacterial species.
METHODS

Strains. Seventy-one strains representing 25 different medically relevant *Corynebacterium* species or subspecies were used in the present study (Table 1). All strains originated from epidemiologically unrelated patients and belonged to the culture collection of the Department of Medical Microbiology, University of Zürich (DMMZ), established by G. Funke. *Turicella otitidis* was included because this genus is the closest phylogenetic neighbour of the genus *Corynebacterium* (Pascual et al., 1995; Ruimy et al., 1995) and the taxon is often confused with *Corynebacterium* species in the routine clinical laboratory (Funke et al., 1997d).

Isolation of DNA. Depending on the growth rate, strains were incubated for 18–43 h at 37 °C on Columbia agar base (Gibco-BRL) supplemented with sheep blood (7%). For the lipophilic species, this medium was also supplemented with 1% Tween 80 (Merck). After checking colony morphology, a cell mass of 30 mg wet weight was scraped from the plate and used for DNA isolation. DNA was isolated by the guanidium thiocyanate method (Pitcher et al., 1989) with the modification of a combined lysozyme and mutanolysin treatment (Björkroth & Korkeala, 1996).

Selection of restriction enzymes for ribotyping. Initially, 15 restriction enzymes, *BamHI, BglII, BssHII, BstEII, Clal, DpnII, DraI, EcoRI, HindIII, SacII, SgrAI, SmaI, SpeI, SphI* and *SspI* (New England Biolabs), were tested for ribotyping of *Corynebacterium–Turicella* by using two strains, *Corynebacterium auris* 328T and *T. otitidis* 272.

Ribotyping. Restriction endonuclease treatment of 2 μg DNA was done as specified by the manufacturer (New

### Table 1. Strains used in the study

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Strain</th>
<th>Taxon</th>
<th>Strain</th>
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<tbody>
<tr>
<td><em>C. accolens</em></td>
<td>DMMZ 1882T</td>
<td><em>C. jeikeium</em></td>
<td>DMMZ 1111T</td>
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<tr>
<td><em>C. afermentans subsp. afermentans</em></td>
<td>DMMZ 1351</td>
<td><em>C. macginleyi</em></td>
<td>DMMZ 1352T</td>
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<tr>
<td><em>C. afermentans subsp. lipophilum</em></td>
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<td><em>C. mucifaciens</em></td>
<td>DMMZ 2278T</td>
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<tr>
<td><em>C. amycolatum</em></td>
<td>DMMZ 1620</td>
<td><em>C. propinquum</em></td>
<td>DMMZ 1184</td>
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<tr>
<td><em>C. argentoratense</em></td>
<td>DMMZ 1619</td>
<td><em>C. pseudodiphtheriticum</em></td>
<td>LA 3518</td>
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<tr>
<td><em>C. auris</em></td>
<td>DMMZ 1336T</td>
<td><em>C. pseudotuberculosis</em></td>
<td>DMMZ 2582</td>
</tr>
<tr>
<td><em>C. confusum</em></td>
<td>DMMZ 1814</td>
<td><em>C. riegelii</em></td>
<td>DMMZ 2582</td>
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<tr>
<td><em>C. coyleae</em></td>
<td>DMMZ 1347</td>
<td><em>C. striatum</em></td>
<td>DMMZ 1527</td>
</tr>
<tr>
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<td><em>C. ulcerans</em></td>
<td>DMMZ 957</td>
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<tr>
<td><em>C. durum</em></td>
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<td><em>C. urealyticum</em></td>
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<td><em>C. xerosis</em></td>
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<td><em>C. xerosis</em></td>
<td>DMMZ 1504T</td>
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<tr>
<td><em>C. imitans</em></td>
<td>DMMZ 2023T</td>
<td><em>T. otitidis</em></td>
<td>DMMZ 272</td>
</tr>
</tbody>
</table>

Strains were obtained from the Culture Collection of the Department of Medical Microbiology, University of Zürich (DMMZ) or the Swiss Culture Collection for Bacteria (LA).
Ribotyping for Corynebacterium identification

Results and Discussion

Of the 15 restriction enzymes tested, BstEII, HindIII, Smal and SphI digestion resulted in good cleavage of DNA from the test strains with revealing ribotypes. These four enzymes were selected for use for the remaining 72 strains. Since the use of HindIII resulted in incomplete digestion with many of the strains, numerical analysis of the strains was performed for BstEII, Smal and SphI ribotypes.

BstEII ribotypes generally had fewer bands (two to 11) (Fig. 1) than the ribotypes obtained with the two other restriction enzymes (Figs 2 and 3). In particular, BstEII cleavage resulted in patterns with few high-molecular-mass fragments. For most species, all strains tested restriction enzymes (Figs 2 and 3). In particular, BstEII, SmaI etc.), whereas, for example, the three strains of Corynebacterium riegelii, C. auris, T. otitidis, and C. minutissimum, C. afermentans subsp. lipophilum and Corynebacterium durum did not cluster together. However, all strains belonging to each of these species, with the exception of C. minutissimum, did cluster together when at least one of the three enzymes was used for ribotyping. Therefore, it is concluded that a combination of the three enzymes is essential in order to identify these medically relevant Corynebacterium species. Since each enzyme provides a different characterization result, no general threshold similarity value can be set within a species. Most of the species exhibited similarity values of 50 to 100% between strains when clustered together (Figs 1–3).

So far, ribotyping of Corynebacterium species has been used mainly for strain tracking or large-scale epidemiological investigations. The majority of studies (applying restriction enzyme BstEII) have focussed on Corynebacterium diphtheriae (De Zoyza et al., 1995; Popovic et al., 1996; Riegel et al., 1997) as the most significant pathogen within the genus Corynebacterium. Further studies used ribotyping (restriction enzymes HindIII, PvuII and BstEII) to demonstrate nosocomial transmission of C. jeikeium (Pitcher et al., 1990) as well as transmission of Corynebacterium imitans between non-hospitalized persons (Funke et al., 1997a). However, ribotyping (with restriction enzymes PvuII and EcoRI) has also been applied successfully in taxonomic descriptions of C. afermentans (Riegel et al., 1993b) and C. propinquum (Riegel et al., 1993a). These authors demonstrated clearly that both C. afermentans and C. propinquum could be identified by ribotyping when multiple restriction enzymes were applied. In a comprehensive study, Soto et al. (1991) observed, based on numerical analysis of rRNA gene patterns (restriction enzymes HindIII and EcoRI), that the former CDC coryneform group D-2 bacteria (synonymous with C. urealyticum) clustered together and represented a taxon separate from the other established corynebacteria. For the non-medical corynebacteria, Liebl et al. (1991) demonstrated that ribotyping (applying restriction
enzymes BamHI, HindIII or BglII) leads to the correct identification of Corynebacterium glutamicum strains.

It remains unclear to us why, despite of the promising results of Liebl et al. (1991) and Soto et al. (1991), the application of ribotyping for Corynebacterium iden-

**Fig. 1.** BstEII dendrogram and ribotypes; left, high molecular mass (23 kbp) and right, low molecular mass (500 bp).
application of ribotyping to identification of Corynebacterium species is only possible after the creation of a comprehensive database, as in the present study. Once this has been done, it is not particularly time-consuming or labour-intensive to screen individual unidentified Corynebacterium strains for their identity. We acknowledge that it may have been desirable to test many more strains of each of the 26 species or subspecies included, but the primary goal of our study was to demonstrate the general applicability of our

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**Fig. 2.** Small dendrogram and ribotypes; left, high molecular mass (23 kbp) and right, low molecular mass (500 bp).
approach for the medically relevant members of the genus *Corynebacterium*. In our view, ribotyping is a complementary method for screening unidentified corynebacteria.

Other established methods used for identifying new *Corynebacterium* species include analysis of the 16S–23S rRNA intragenic spacer region (Aubel et al., 1997) and the use of whole-cell protein profiles (Sjödén

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**Fig. 3.** Sphi dendrogram and ribotypes; left, high molecular mass (23 kbp) and right, low molecular mass (500 bp).
et al., 1998). Species delineation within the genus Corynebacterium has often been achieved by sequencing of full 16S rRNA genes (Pascual et al., 1995; Ruimy et al., 1995), because the genus exhibits an enormous phylogenetic depth. Compared to other genera, 16S rDNA divergence rates are relatively high within the genus Corynebacterium (Pascual et al., 1995; Ruimy et al., 1995), which allows new Corynebacterium species to be established on the basis of 16S rRNA gene sequences. However, if sequencing facilities are not available, we consider ribotyping as a useful initial approach to test whether an unidentified Corynebacterium strain represents a new species.

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REFERENCES


