Identification of the emerging skin pathogen *Corynebacterium amycolatum* using PCR-amplification of the essential *divIVA* gene as a target

M. Letek[1], E. Ordóñez[1], I. Fernández-Natal[2], J.A. Gil[1] & L.M. Mateos[1]

[1]Departamento de Ecología, Genética y Microbiología, Área de Microbiología, Facultad de Biología, Universidad de León, León, Spain; and
[2]Departamento de Microbiología, Complejo Hospitalario de León, Altos de Nava, León, Spain

Correspondence: Luís M. Mateos, Departamento de Ecología, Genética y Microbiología, Área de Microbiología, Facultad CC, Biológicas y Ambientales, Universidad de León, 24071 León, Spain. Tel.: +34 987 291126; fax: +34 987 291409; e-mail: deglmd@unileon.es

Received 22 September 2006; accepted 2 October 2006.
First published online 27 October 2006.

DOI:10.1111/j.1574-6968.2006.00492.x

Editor: Jeff Cole

Keywords
Corynebacterium; PCR; *divIVA*; ARDRA; 16S rRNA gene; real-time PCR.

Abstract

The actinomycete *Corynebacterium amycolatum* is a saprophytic bacterium usually associated with the human skin, but it is at present considered an emergent pathogen as it is isolated from nosocomial settings from samples of immunosuppressed patients. The conventional method to distinguish *C. amycolatum* from closely related species is mainly based on phenotypic or chemotaxonomic studies. We developed a molecular method to identify rapidly *C. amycolatum* based on the use of different primers for amplification of the cell division *divIVA* gene using conventional or real-time PCR. This technique was used for the first time to distinguish *C. amycolatum* from the closely related *Corynebacterium striatum*, *Corynebacterium minutissimum* and *Corynebacterium xerosis*, without the requirement of further molecular analysis. The suitability of the identification method was tested on 51 clinical isolates belonging to the nonlipophilic fermentative group of corynebacteria (cluster *C. striatum/C. amycolatum*), which were accurately characterized by sequencing a 0.8 kb fragment of the 16S rRNA gene.

Introduction

The genus *Corynebacterium* includes a broad group of pathogenic species, in which the most prominent member is *Corynebacterium diphtheriae*, the pathogenic agent of diphtheria. However, in the last few years, interest in clinical microbiology of the so-called nondiphtherial corynebacteria has increased due to the emergence of multidrug-resistant pathogens belonging to this group of bacteria (Otsuka *et al.*, 2006). This concern has led recently to the sequencing of the *Corynebacterium jeikeium* genome (Tauch *et al.*, 2005), which, together with *Corynebacterium urealyticum*, is now considered to be clearly associated with human diseases (Fernández-Natal *et al.*, 2001).

Several nonlipophilic members of this broad group of pathogenic corynebacteria have recently been reported to be involved in a rising number of recognized infections (von Graevenitz *et al.*, 1998). One of them, the mycolic acid-less corynebacterium *Corynebacterium amycolatum* isolated from human skin (Collins *et al.*, 1988), is frequently associated with clinical samples from immunosuppressed patients (de Miguel-Martinez *et al.*, 1996; Esteban *et al.*, 1999; Oteo *et al.*, 2001). Related corynebacteria such as *Corynebacterium xerosis*, *Corynebacterium minutissimum* and *Corynebacterium striatum*, along with the aforementioned species, are the most common *Corynebacterium* isolates from clinical samples (Renaud *et al.*, 1998). The increasing antibiotic resistance associated with some of these clinical isolates (de Miguel-Martinez *et al.*, 1996) makes it important to resolve the frequent misclassification problems of these species (Funke *et al.*, 1996; Wauters *et al.*, 1996; Oteo *et al.*, 2001).

Infections by *Corynebacterium* species are conventionally diagnosed by culturing, followed by phenotypic analysis of the isolates (API Coryne strips, BioMérieux) (Funke *et al.*, 1997a). However, the reliability of the identification of *Corynebacterium* isolates by their phenotype is limited and in some cases only 25–30% of the samples were correctly identified (Roux *et al.*, 2004). Identification of *Corynebacterium* species on the basis of chemotaxonomic (Voisin *et al.*, 2004) or molecular analysis (Vaneechoutte *et al.*, 1995; Khamis *et al.*, 2004; Roux *et al.*, 2004; Khamis *et al.*, 2005) is possible, but in many cases these methods are arduous and lengthy for routine use in clinical laboratories. Sequencing of the 16S rRNA genes is a recognized 'gold standard' and hence these targets may be used as 'molecular clocks' (Woese, 1987). However, sequencing is expensive and time consuming, and

therefore improvements in genetic methods for precise species identification such as real-time PCR offer a viable alternative for pathogenic bacteria (Espy *et al.*, 2006).

We have used *Corynebacterium glutamicum* as a nonpathogenic model for the identification and characterization of potential antimicrobial targets (Ramos *et al.*, 2003; Ramos *et al.*, 2005; Valbuena *et al.*, 2006). The *divIVA* gene is essential for cell growth in *C. glutamicum* (Ramos *et al.*, 2003). It has been the subject of special attention in our studies as it presents some highly conserved and variable regions throughout its nucleotide sequence. In this work, we describe a new identification strategy based on the PCR amplification of a *divIVA* gene-fragment for the rapid identification of *C. amycolatum* vs. other closely related species. We have checked the accuracy of this molecular tool with 51 clinical samples belonging to the nonlipophilic fermentative group of corynebacteria that were previously identified by phenotypic and molecular [amplified rDNA-restriction analysis (ARDRA) and rRNA gene sequencing] analysis.

Materials and methods

Strains, media and culture conditions

Wild-type strains used for this work were *C. amycolatum* CECT 4163 (ATCC 49368), *C. glutamicum* ATCC 13032, *C. jeikeium* CECT 760 (ATCC 33031), *C. minutissimum* CECT 4158 (ATCC 23348), *C. striatum* CECT 4159 (ATCC 6940) and *C. xerosis* CECT 4160 (ATCC 373). The 51 clinical *Corynebacterium* isolates were collected at the Microbiology Service of the Hospital of León (Spain) during a period of 3 years (from 2000 to 2002) as unique bacteria present in the clinical sample. The origin of the 51 isolates was as follows: 27 from blood samples, 13 from central vascular catheters and 11 from corporal exudates. Bacteria from blood samples were isolated from the aerobic bottles of the automated blood culture system BacT/Alert 3D microbial detection system (BioMérieux). Vascular catheters and exudate samples as well as the control strains were cultured aerobically at 37 °C on Sheep Blood Agar (SBA, BD Diagnostic System). For protein isolation, wild-type strains were propagated at 37 °C in 500 mL Erlenmeyer flasks containing 100 mL of Trypticase Soy Broth, with an agitation rate of 200 r.p.m. The clinical significance of the samples was determined following the criteria described previously (Funke *et al.*, 1997b).

Morphological and biochemical analysis

The identification of the isolates as members of the genus *Corynebacterium* was based on: (i) standard bacteriological techniques, including colony morphology (Funke *et al.*, 1997b); (ii) Gram staining to determine their cell shape and whether they belong to the Gram-positive rods group; and (iii) the system API Coryne database 2.0 (Funke *et al.*, 1997a). Isolated colonies were grown on SBA medium for 24 h at 37 °C, and a cell suspension was obtained using the corresponding McFarland standard to inoculate the API Coryne strips following the manufacturer's instructions (BioMérieux). The strips were automatically read in an ATB 1525 Reader (BioMérieux) after 24 and 48 h of incubation. A sample of each isolate was streaked on an SBA plate and incubated at 37 °C for 48 h to analyse the macroscopic colony-character.

Nucleic acid isolation and manipulation

Total DNA was extracted by boiling using a method described previously for *Corynebacterium* (Vaneechoutte *et al.*, 1995). Briefly, a loopful of colony (10 μL) grown on SBA was resuspended in 300 μL of water, boiled for 10 min, centrifuged for 2 min and the supernatant was used for further analysis. The 16S rRNA genes were amplified as a 0.8 kb fragment using the FastStart High Fidelity PCR System (Roche) and the standard oligonucleotides 8FPL (gcggatccgcggccgctgcagagtttgatcctggctcag) and 806R (gcggatccgcggccgcggactaccagggtatctaat) (Relman, 1993), under the following PCR conditions. DNA samples of known concentration were first denatured at 95 °C for 5 min and subjected to 30 amplification cycles (denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 1 min), finishing with an extra cycle at 72 °C for 10 min. The 0.8 kb PCR-amplified fragments (rRNA gene) from the 51 clinical isolates were used directly as a template for sequencing using the primers 8FPL and 806R or for ARDRA analysis using the restriction endonucleases *AluI*, *RsaI*, *CfoI* and *HaeIII* (from New England Biolabs).

For *divIVA* amplification, two different primer pairs were used: div1 (ggaattcatgccgttgactccagc)/div2 (ggaattccggtactcacgctcgaa) and div1/ileS (ggacatatctacgcg), in order to amplify two overlapping fragments of 1 and 1.7 kb, respectively, using the following PCR conditions. DNA samples of known concentration were first denatured at 95 °C for 5 min and subjected to 30 amplification cycles (denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 1 min) and a final cycle at 72 °C for 10 min. The amplified bands were cloned into pGEM-TEasy (Promega), using *Escherichia coli* TOP10 (Invitrogen) as a host for transformation, and sequenced.

For real-time PCR, DNA samples were used as a template for PCR amplification with 12.5 μL of master mix reagent (Applied Biosystems), and up to 25 μL of water. Reactions were performed with an ABI Prism 7000 sequence detection system (Applied Biosystems) with the following program: 2 min at 50 °C; 10 min at 95 °C; and 50 cycles of 15 s at 95 °C and 1 min at 60 °C. The results obtained by interpolation in a standard regression curve of cycle threshold (C_T) values generated from samples of known DNA concentration were

processed using specific software (ABI PRISM 7000 SDS software). C_T is defined as the cycle at which fluorescence is determined to be statistically significant above background and is inversely proportional to the log of the initial copy number. Real-time PCRs with C_T values of > 40 were considered negative.

DNA sequencing and bioinformatic tools

The DNA sequencing was performed using the dideoxy nucleotide chain termination method of Sanger and MegaBACE 1500 System (GE Healthcare). DNA sequences were analysed with DNASTAR (DNAstar, Inc., London, UK) and similarity searches were performed at the BLAST and FASTA public servers (NCBI, Bethesda, MD). Phylogenetic analyses were processed using MEGA3 (Molecular Evolutionary Genetics Analysis Software) (Kumar *et al.*, 2004) with the neighbour-joining method.

Preparation of cell-free extracts, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

For *Corynebacterium* cell-free extract preparation, 10 mL of cell culture with $OD_{600\,nm} = 1$ was pelleted and resuspended in 600 μL of phosphate buffered saline (PBS). Cells were broken using the FastPROTEIN Blue Lysing Matrix (Qbiogene Inc.) and the BIO101 Thermo Savant FastPrep FP120 (Qbiogene Inc.) for two periods of 20 s at a power setting of 6.0, with a 5-min interval in ice-cooled tubes. Cell debris was removed by centrifugation at 10000 ***g*** for 1 min at 4 °C, and the supernatants were used as cell extracts. SDS-PAGE of cell extracts from different microorganisms was performed essentially as described (Laemmli, 1970), using 1 μg of total protein per lane. Electrophoresis was achieved at room temperature in a vertical slab gel ($170 \times 130 \times 1.5$ mm) using 10% (w/v) polyacrylamide at 100 V and 60 mA. After electrophoresis, proteins were electroblotted to polyvinylidene difluoride membranes (Millipore) and immunostained using mouse monoclonal antibodies (F126-2) raised against purified Antigen 84/DivIVA from *Mycobacterium kansasii* (kindly provided by Prof. A.H.J. Kolk, Royal Tropical Institute, Amsterdam, the Netherlands) at 1 : 10 000 dilution; polyclonal antimouse IgG-AP antibodies (Santa Cruz Biotechnology) at 1 : 10 000 dilution were used as secondary antiserum.

Nucleotide sequence accession numbers

divIVA genes from *C. amycolatum* and *C. xerosis* were incorporated into the EMBL nucleotide sequence database with the accession numbers AM261216 and AM286228, respectively.

Results

Heterogeneity of DivIVA in different type strains of corynebacteria

In our previous work, we reported the detection of DivIVA from *C. glutamicum* (Ramos *et al.*, 2003) using monoclonal antibodies raised against Antigen 84 (DivIVA) from *M. kansasii* (Hermans *et al.*, 1995). In this work, we attempted to confirm the presence of the DivIVA protein in *C. amycolatum* and other closely related bacteria. Western blot assays were achieved using crude extracts from the type strains *C. glutamicum, C. amycolatum, C. striatum, C. jeikeium, C. minutisimum* and *C. xerosis*. All of the analysed strains gave a positive reaction with anti-Antigen 84 antibodies as expected for an essential protein, although variability in band size was evident (Fig. 1a). The different size was in agreement with the size heterogeneity of the *divIVA* genes from the coryneform species whose genomes have been sequenced [*C. glutamicum* (accession number NC003450), *Corynebacterium efficiens* (NC004369), *C. diphtheriae* (NC002935) and the recently incorporated *C. jeikeium* (NC007164)].

In addition, we performed alignment analysis of the aforementioned *divIVA* sequences and we observed highly conserved (at the 5′ and 3′ ends) and variable (internal) regions (Fig. 1b). Based on these results, we expected that the *divIVA*-associated polymorphism could be used as a 'molecular clock' to distinguish species from related bacteria, and in particular, for members of corynebacteria.

From the indicated alignment of the *divIVA* genes, we defined the primers div1 and div2 that were complementary just to the 5′ end (div1) and to a region close to the 3′ end (div2) of *divIVA* (Fig. 1b). An additional primer, ileS, complementary to a conserved region of the *ileS* gene that was always located downstream of *divIVA* in coryneform species, was also used for amplification purposes (see Fig. 1b).

PCR amplification using the primer pair div1/div2 and total DNA of the type strains *C. glutamicum*, *C. amycolatum*, *C. xerosis*, *C. striatum* and *C. minutissinum* as a template allowed us to amplify a 1-kb band only in samples corresponding to *C. glutamicum* and *C. amycolatum* (Fig. 2a). Moreover, when we used the primer pair div1/ileS for amplification, a 1.7 kb band was observed in samples from *C. glutamicum*, *C. amycolatum* and *C. xerosis* (Fig. 2a). Therefore, the 1.7 kb fragments containing the full *divIVA* genes (and downstream sequences) from *C. amycolatum* and *C. xerosis* were cloned into the vector pGEM-TEasy, sequenced and deposited at the EMBL database with accession numbers AM261216 and AM286228, respectively.

As real-time PCR analysis is frequently applied for accurate and fast identification of pathogenic microorganisms (Espy *et al.*, 2006), we used this methodology with total DNA obtained from the aforementioned wild-type strains.

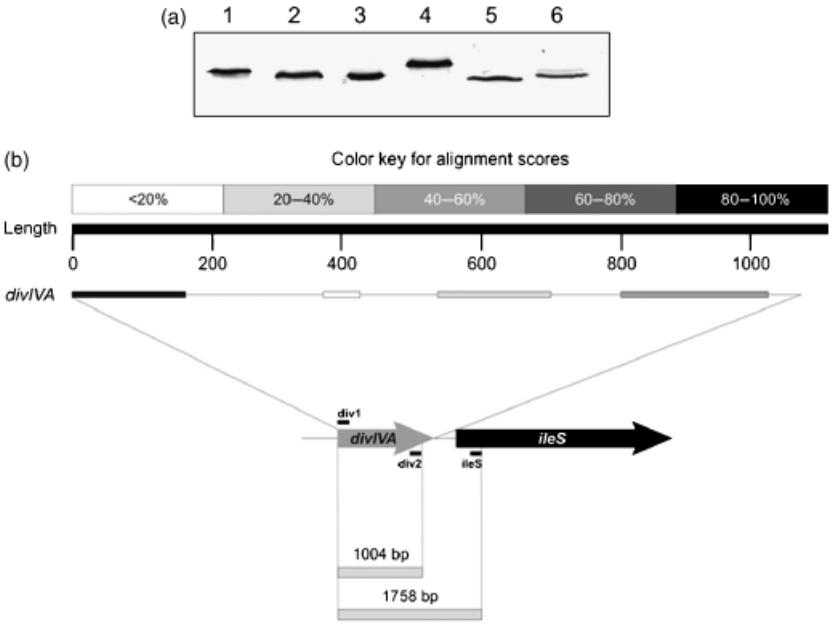

Fig. 1. (a) Detection of DivIVA using monoclonal anti-Antigen 84 antibodies in different cell extracts from the following *Corynebacterium* type strains: lane 1, *Corynebacterium glutamicum*; lane 2, *Corynebacterium amycolatum*; lane 3, *Corynebacterium striatum*; lane 4, *Corynebacterium jeikeium*; lane 5, *Corynebacterium minutissimum*; lane 6, *Corynebacterium xerosis*. (b) *divIVA* gene from *Corynebacterium* showing the conserved and variable regions in its sequence. A diagram of the primers used in this study is shown at the bottom; the primer pairs div1/div2 and div1/ileS amplify bands of 1004 and 1758 bp (respectively).

Results comparable to those previously found by conventional PCR were obtained when primer pairs div1/div2 or div1/ileS were used. With the former primer pair, amplification signals corresponding to *C. glutamicum* and *C. amycolatum* were observed (Fig. 2b) with C_T values of 22 ± 0.03 and 25 ± 0.05, respectively. PCR amplification with DNA from strains *C. striatum*, *C. minutisimum* and *C. xerosis* was unsuccessful (see the partial lines in Fig. 2b) even after repeated attempts with varying conditions of amplification. When primers div1/ileS were used, amplification curves were observed for *C. glutamicum* ($C_T = 30 \pm 0.05$), *C. amycolatum* ($C_T = 32.4 \pm 0.07$) and *C. xerosis* ($C_T = 32.5 \pm 0.08$) (Fig. 2c).

Morphological and biochemical characteristics of the clinical isolates

Gram staining of the 51 clinical isolates revealed Gram-positive and a typical corynebacterial cell shape in all cases. The macroscopic colony characteristics were seen after 48 h of incubation at 37 °C in SBA medium. Most of the strains presented rough, dry and whitish colonies with irregular margins and a diameter of 1–1.5 mm, without specific macroscopic distinctions to each other. However, eight of the isolates showed some morphological differences, five of which presented the same general characters except for a smaller colony size, whereas the three remaining isolates (numbers 10, 37 and 43) formed round, smooth and shiny colonies. The API Coryne strip was used for species determination of the 51 clinical isolates, and the main codes were 310032(4/5) corresponding to an identification of members from the *C. striatum*/*C. amycolatum* nonlipophilic fermentative group. The variability at the last digit was due to differences in sucrose fermentation (4/5). Most of the isolates had the enzymes for the nitrate metabolism, pyrazinamidase and alkaline phosphatase but not for pyrrolidonyl arylamidase, β-glucuronidase, β-galactosidase, *N*-acetyl β-glucosamidase, aesculinase (β-glucosidase) and urease. They did not hydrolyse gelatin. They fermented glucose, ribose, maltose and sucrose (the last one occasionally) according to the API Coryne gallery.

Molecular analysis of the human clinical samples

Total DNA obtained from the 51 clinical samples was used to amplify both the 16S rRNA and *divIVA* genes by PCR and/or real-time PCR. In 48 cases, the ARDRA analysis of the 0.8 kb rRNA gene amplified fragments (using primers 8FPL/806R) was in agreement with the ARDRA pattern indicated for *C. amycolatum*, but isolates 10, 37 and 43 showed patterns corresponding to *C. minutissimum* (isolate 10) and *C. striatum* (isolates 37 and 43). Sequencing of the 0.8 kb rRNA gene fragments from all the 51 clinical samples corroborated that isolates 37 and 43 belonged to *C. striatum*, but isolate 10 was much more related to *Corynebacterium aurimucosum* than to *C. minutissimum*. Recently, it was reported that the last two strains could be differentiated, although they are very closely related (Yassin *et al.*, 2002). For the remaining clinical isolates, the corresponding 0.8 kb fragment sequence was 99–100% identical to that described for the *C. amycolatum* wild-type strain.

divIVA amplification (by conventional PCR or real-time PCR) using primers div1/div2 or div1/ileS was successful for

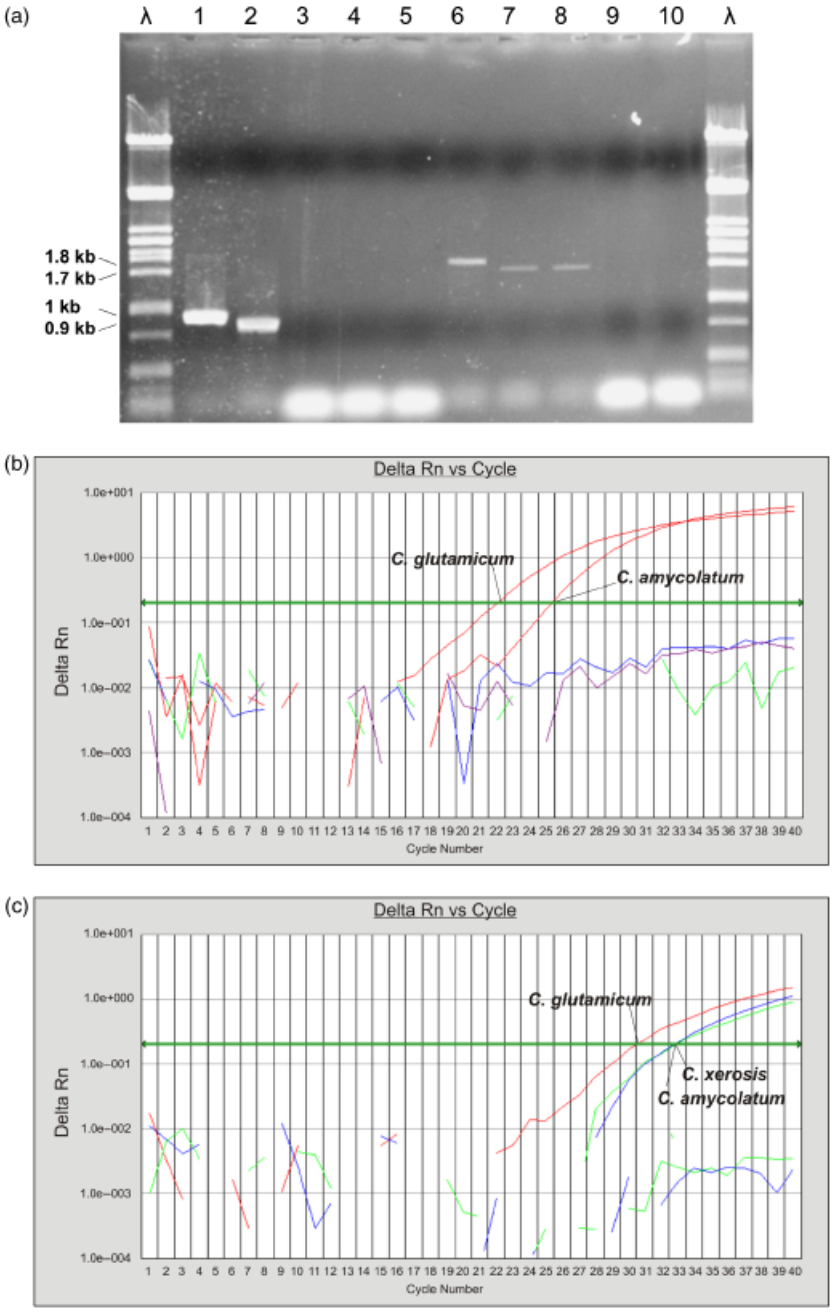

Fig. 2. (a) Conventional PCR amplifications. Lanes 1–5, using the primer pair div1/div2; lanes 6–10, using the primer pair div1/ileS. Total DNAs from different *Corynebacterium* type strains: lanes 1 and 6, *Corynebacterium glutamicum*; lanes 2 and 7, *Corynebacterium amycolatum*; lanes 3 and 8, *Corynebacterium xerosis*; lanes 4 and 9, *Corynebacterium striatum*; lanes 5 and 10, *Corynebacterium minutissimum*. λ, Lambda DNA digested with *Pst*I. (b) Real-time PCR assay using the primer pair div1/div2 and total DNA from *Corynebacterium* type strains. The C_T values were determined in all the cases using the same quantity of DNA per sample. Amplification from only *C. glutamicum* and *C. amycolatum* DNA was detected. (c) Real-time PCR assay using the primer pair div1/ileS and total DNA from *Corynebacterium* type strains. In this case, amplification was detected from *C. glutamicum*, *C. amycolatum* and *C. xerosis* DNA.

the 48 clinical isolates, confirming that they were indeed *C. amycolatum* strains. As expected, *divIVA* was not amplified with total DNA obtained from clones 10, 37 and 43. As an internal positive control, we obtained real-time PCR amplification for the 0.8 kb rRNA gene fragment in all of the clinical isolates (including clones 10, 37 and 43, data not shown). The lack of amplification using primers div1/div2 or div1/ileS in samples 10, 37 and 43 was therefore due to the fact that they were not *C. amycolatum*, as shown previously when ARDRA and sequencing analysis of the rRNA gene from these clones were achieved (see above).

Discussion

Among the clinical isolates of catalase-positive coryneform organisms, *C. amycolatum* is clearly one of the most frequent and, together with *C. jeikeium* and *C. urealyticum*, constitute one of the most antibiotic-resistant groups of

corynebacteria (Lagrou *et al.*, 1998; Fernández-Natal *et al.*, 2001). For this reason, accurate identification of the coryneform clinical isolates is important for treatment purposes. In many cases, this can be difficult because the conventional methods used for phenotypic recognition (biochemical and morphological analysis) are not sufficient for identification of *Corynebacterium* species (Renaud *et al.*, 1998; Roux *et al.*, 2004), and therefore molecular analysis should be applied.

In this work, we have shown that it is possible to differentiate closely related nonlipophilic *Corynebacterium* species by PCR using *divIVA* as a target. Amplification of *divIVA* using the primer pairs div1/div2 or div1/ileS was successful when DNA from *C. amycolatum* was included, but no amplification was obtained when DNA from the very closely related species *C. striatum* and *C. minutissimum* were present. Amplification using the same primer pairs was also obtained from the *C. glutamicum* type strain; however, this strain has never been described to be involved in pathogenic events and therefore it would be absent from clinical samples. In addition, this molecular method could be used for the identification of *C. xerosis* clinical isolates as we observed successful amplification only with the primer pair div1/ileS; however, nowadays, the presence of *C. xerosis* in human clinical samples is uncommon (Funke *et al.*, 1996). The recent descriptions of new nonlipophilic *Corynebacterium* species such as *Corynebacterium tuscaniae* (Riegel *et al.*, 2006), *Corynebacterium sundsvallense* (Collins *et al.*, 1999), *Corynebacterium riegelii* (Funke *et al.*, 1998), *Corynebacterium thomssenii* (Zimmermann *et al.*, 1998) or *Corynebacterium freneyi* (Renaud *et al.*, 2001) were not taken into account in the present work as they have little relevance at the clinical level.

An unexpected difficulty was encountered when we attempted the phenotypic identification of the 51 clinical samples analysed in this study. Using the API Coryne strips, all of the strains were identified as members of the *C. striatum*/*C. amycolatum* group. Three of them exhibited colony morphology different from *C. amycolatum*, and their identity as a different species was confirmed by rRNA gene sequencing. However, the other 48 isolates were identified as *C. amycolatum*, as was confirmed by *divIVA* amplification and corroborated by sequencing of the rRNA gene fragment. Five of these *C. amycolatum* isolates formed smaller colonies than the wild-type strain, although they were in fact *C. amycolatum*. The small colony size of these isolates could be part of a general strategy used by certain bacteria to facilitate the infection processes (Proctor *et al.*, 2006). This makes them more elusive for the correct identification by clinical microbiologists.

During the last decade, different molecular methods have been used to achieve a faster but precise identification of *Corynebacterium* species: (i) the ARDRA analysis was used to differentiate *Corynebacterium* species (Vaneechoutte *et al.*, 1995) or to differentiate *Corynebacterium bovis* from other members of the lipophilic group (Huxley *et al.*, 2004); (ii) PCR-RFLP to differentiate *C. amycolatum* from *C. striatum* (Sierra *et al.*, 2005); (iii) sequencing of rRNA genes (Roux *et al.*, 2004) or an *rpoB* gene fragment (Khamis *et al.*, 2004; Khamis *et al.*, 2005). However, all of these methods are time consuming and at least a two-step protocol involving PCR amplification and further digestion and/or sequencing (with the necessary analysis in both cases) is required.

Here we analysed the usefulness of specific PCR-amplifications of the *divIVA* gene from *C. amycolatum*, which has been sequenced in this work, for the rapid identification of this emergent pathogenic microorganism without the need for the laborious and lengthy digestion and/or sequencing of

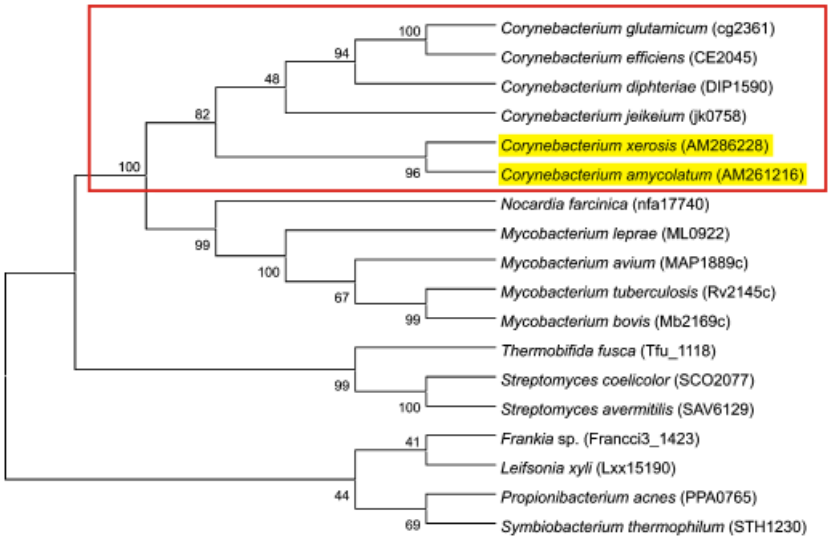

Fig. 3. Phylogenetic analysis of DivIVA proteins from 18 different actinomycetes using the neighbour-joining method.

an amplified DNA fragment. A phylogenetic tree of different DivIVA proteins from actinomycetes (including those from *C. amycolatum* and *C. xerosis*) is shown in Fig. 3, confirming the suitability of DivIVA as a 'molecular clock'. The special properties of *divIVA* from corynebacteria, such as its essentiality for growth and the presence of conserved internal regions for species-specific amplification, make this target a good choice for molecular discrimination.

Acknowledgements

This work was funded by grants from the Diputación de León (ref. 2004/120) and the Fondo de Investigaciones Sanitarias (ref. PI030534). Michal Letek is a recipient of a fellowship from the FPU (Ministry of Education) and Efrén Ordóñez from the Junta de Castilla y León. We acknowledge Dr A.H.J. Kolk (Royal Tropical Institute, Amsterdam, the Netherlands) for the monoclonal antibody against Antigen 84/DivIVA. The critical reading of the manuscript by Prof. F. Gudiol (Servicio de Enfermedades Infecciosas, Hospital Universitario de Bellvitge, Barcelona, Spain) and Dr Vasant K. Chary (Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania) is gratefully acknowledged.

References

Collins MD, Burton RA & Jones D (1988) *Corynebacterium amycolatum* sp. nov., a new mycolic acid-less *Corynebacterium* species from human skin. *FEMS Microbiol Lett* **49**: 349–352.

Collins MD, Bernard KA, Hutson RA, Sjoden B, Nyberg A & Falsen E (1999) *Corynebacterium sundsvallense* sp. nov., from human clinical specimens. *Int J Syst Bacteriol* **49**: 361–366.

de Miguel-Martinez I, Fernandez-Fuertes F, Ramos-Macias A, Bosch-Benitez JM & Martin-Sanchez AM (1996) Sepsis due to multiply resistant *Corynebacterium amycolatum*. *Eur J Clin Microbiol Infect Dis* **15**: 617–618.

Espy MJ, Uhl JR, Sloan LM *et al.* (2006) Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin Microbiol Rev* **19**: 165–256.

Esteban J, Nieto E, Calvo R, Fernandez-Roblas R, Valero-Guillen PL & Soriano F (1999) Microbiological characterization and clinical significance of *Corynebacterium amycolatum* strains. *Eur J Clin Microbiol Infect Dis* **18**: 518–521.

Fernandez-Natal I, Guerra J, Alcoba M, Cachon F & Soriano F (2001) Bacteremia caused by multiply resistant *Corynebacterium urealyticum*: six case reports and review. *Eur J Clin Microbiol Infect Dis* **20**: 514–517.

Funke G, Lawson PA, Bernard KA & Collins MD (1996) Most *Corynebacterium xerosis* strains identified in the routine clinical laboratory correspond to *Corynebacterium amycolatum*. *J Clin Microbiol* **34**: 1124–1128.

Funke G, Renaud FN, Freney J & Riegel P (1997a) Multicenter evaluation of the updated and extended API (RAPID) Coryne database 2.0. *J Clin Microbiol* **35**: 3122–3126.

Funke G, von Graevenitz A, Clarridge JE & Bernard KA (1997b) Clinical microbiology of coryneform bacteria. *Clin Microbiol Rev* **10**: 125–159.

Funke G, Lawson PA & Collins MD (1998) *Corynebacterium riegelii* sp. nov., an unusual species isolated from female patients with urinary tract infections. *J Clin Microbiol* **36**: 624–627.

Hermans PW, Abebe F, Kuteyi VI, Kolk AH, Thole JE & Harboe M (1995) Molecular and immunological characterization of the highly conserved antigen 84 from *Mycobacterium tuberculosis* and *Mycobacterium leprae*. *Infect Immun* **63**: 954–960.

Huxley JN, Helps CR & Bradley AJ (2004) Identification of *Corynebacterium bovis* by endonuclease restriction analysis of the 16S rRNA gene sequence. *J Dairy Sci* **87**: 38–45.

Khamis A, Raoult D & La Scola B (2004) *rpoB* gene sequencing for identification of *Corynebacterium* species. *J Clin Microbiol* **42**: 3925–3931.

Khamis A, Raoult D & La Scola B (2005) Comparison between *rpoB* and 16S rRNA gene sequencing for molecular identification of 168 clinical isolates of *Corynebacterium*. *J Clin Microbiol* **43**: 1934–1936.

Kumar S, Tamura K & Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**: 150–163.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Lagrou K, Verhaegen J, Janssens M, Wauters G & Verbist L (1998) Prospective study of catalase-positive coryneform organisms in clinical specimens: identification, clinical relevance, and antibiotic susceptibility. *Diagn Microbiol Infect Dis* **30**: 7–15.

Oteo J, Aracil B, Ignacio AJ & Luis Gomez-Garces J (2001) Significant bacteremias by *Corynebacterium amycolatum*: an emergent pathogen. *Enferm Infec Microbiol Clin* **19**: 103–106.

Otsuka Y, Ohkusu K, Kawamura Y, Baba S, Ezaki T & Kimura S (2006) Emergence of multidrug-resistant *Corynebacterium striatum* as a nosocomial pathogen in long-term hospitalized patients with underlying diseases. *Diagn Microbiol Infect Dis* **54**: 109–114.

Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M & Peters G (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* **4**: 295–305.

Ramos A, Honrubia MP, Valbuena N, Vaquera J, Mateos LM & Gil JA (2003) Involvement of DivIVA in the morphology of the rod-shaped actinomycete *Brevibacterium lactofermentum*. *Microbiology* **149**: 3531–3542.

Ramos A, Letek M, Campelo AB, Vaquera J, Mateos LM & Gil JA (2005) Altered morphology produced by *ftsZ* expression in *Corynebacterium glutamicum* ATCC 13869. *Microbiology* **151**: 2563–2572.

Relman DA (1993) Universal bacterial 16S rDNA amplification and sequencing. In: *Diagnostic molecular microbiology; principles and applications*. (Persing DH, Smith TF, Tenover FC & White TJ, eds), pp. 489–495. Mayo Foundation/American Society for Microbiology, Rochester, MN/Washington, DC.

Renaud FN, Dutaur M, Daoud S, Aubel D, Riegel P, Monget D & Freney J (1998) Differentiation of *Corynebacterium amycolatum*, *C. minutissimum*, and *C. striatum* by carbon substrate assimilation tests. *J Clin Microbiol* **36**: 3698–3702.

Renaud FN, Aubel D, Riegel P, Meugnier H & Bollet C (2001) *Corynebacterium freneyi* sp. nov., alpha-glucosidase-positive strains related to *Corynebacterium xerosis*. *Int J Syst Evol Microbiol* **51**: 1723–1728.

Riegel P, Creti R, Mattei R, Nieri A & von Hunolstein C (2006) Isolation of *Corynebacterium tuscaniae* sp. nov. from blood cultures of a patient with endocarditis. *J Clin Microbiol* **44**: 307–312.

Roux V, Drancourt M, Stein A, Riegel P, Raoult D & La Scola B (2004) *Corynebacterium* species isolated from bone and joint infections identified by 16S rRNA gene sequence analysis. *J Clin Microbiol* **42**: 2231–2233.

Sierra JM, Martinez-Martinez L, Vazquez F, Giralt E & Vila J (2005) Relationship between mutations in the *gyrA* gene and quinolone resistance in clinical isolates of *Corynebacterium striatum* and *Corynebacterium amycolatum*. *Antimicrob Agents Chemother* **49**: 1714–1719.

Tauch A, Kaiser O, Hain T *et al.* (2005) Complete genome sequence and analysis of the multiresistant nosocomial pathogen *Corynebacterium jeikeium* K411, a lipid-requiring bacterium of the human skin flora. *J Bacteriol* **187**: 4671–4682.

Valbuena N, Letek M, Ramos A, Ayala J, Nakunst D, Kalinowski J, Mateos LM & Gil JA (2006) Morphological changes and proteome response of *Corynebacterium glutamicum* to a partial depletion of FtsI. *Microbiology* **152**: 2491–2503.

Vaneechoutte M, Riegel P, de Briel D, Monteil H, Verschraegen G, de Rouck A & Claeys G (1995) Evaluation of the applicability of amplified rDNA-restriction analysis (ARDRA) to identification of species of the genus *Corynebacterium*. *Res Microbiol* **146**: 633–641.

Voisin S, Terreux R, Renaud FN, Freney J, Domard M & Deruaz D (2004) Pyrolysis patterns of 5 close *Corynebacterium* species analyzed by artificial neural networks. *Antonie Van Leeuwenhoek* **85**: 287–296.

von Graevenitz A, Punter-Streit V, Riegel P & Funke G (1998) Coryneform bacteria in throat cultures of healthy individuals. *J Clin Microbiol* **36**: 2087–2088.

Wauters G, Driessen A, Ageron E, Janssens M & Grimont PAD (1996) Propionic acid-producing strains previously designated as *Corynebacterium xerosis*, *C. minutissimum*, *C. striatum*, and CDC group I2 and group F2 coryneforms belong to the species *Corynebacterium amycolatum*. *Int J Syst Bacteriol* **46**: 653–657.

Woese CR (1987) Bacterial evolution. *Microbiol Rev* **51**: 221–271.

Yassin AF, Steiner U & Ludwig W (2002) *Corynebacterium aurimucosum* sp. nov. and emended description of *Corynebacterium minutissimum* Collins and Jones (1983). *Int J Syst Evol Microbiol* **52**: 1001–1005.

Zimmermann O, Sproer C, Kroppenstedt RM, Fuchs E, Kochel HG & Funke G (1998) *Corynebacterium thomssenii* sp. nov., a *Corynebacterium* with *N*-acetyl-beta-glucosaminidase activity from human clinical specimens. *Int J Syst Bacteriol* **48**: 489–494.