

**The development and evaluation of PCR-based DNA-fingerprinting techniques
for the identification of cultured bacteria and fungi
in the routine clinical microbiology laboratory**

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I. Scope of the study

I.1. The four subdivisions of clinical microbiology.

During the nineteenth century Koch's postulates provided the criteria to confirm the germ theory of Pasteur indicating that microorganisms are the cause of several human and animal diseases.

Following these discoveries the field of clinical microbiology was established.

The field of clinical microbiology can be split into four subdivisions based on the nature of the microorganisms investigated.

Historically microbiology started in 1677 with the discovery of bacteria under the microscope of Antonie van Leeuwenhoek. Bacteria are prokaryotic organisms, present in every environmental condition on Earth. Some of these are able to infect the human body and these organisms are the interest of clinical bacteriological testing.

Secondly there is virology, the study of the viruses. Viruses are a totally different biological entity unable to replicate autonomously. They are not living cells because they only possess genomic material (DNA or RNA) and proteins but lack ribosomes and have no cell membrane. Therefore they depend on the infected cell's biosynthetic machinery for protein synthesis. This dependence on the infected host, makes viruses obligate parasites.

A third division is mycology, the study of fungi, which are eukaryotic organisms known as yeasts (unicellular) and molds (multicellular). Fungi have an important mutual characteristic: they are saprophytic pathogens not ingesting food, but absorbing dissolved nutrients from the environment. Only a few are of clinical importance, like the dermatophytes infecting hair and skin and the members of the genera *Candida* and *Aspergillus*.

Finally there is parasitology, which can be separated in two groups. The first is the micro-parasitology, studying protozoa, which are unicellular eukaryotic microorganisms that lack a cell wall. They are generally colourless and motile. Two well-known diseases are malaria and toxoplasmosis caused by respectively *Plasmodium falciparum* and *Toxoplasma gondii*.

Secondly there are the multicellular parasites, which are larger but are historically included in clinical microbiology, because the diagnosis is made on microscopic investigation of samples on the presence of e.g. their eggs. Examples are worms (nematodes (roundworms), trematodes

(flukes) and cestodes (tapeworms)) and small insects like lice (*Pediculosis capitis* and *Pediculosis pubis*) and mites (*Sarcoptes scabiei*).

This doctoral work is focused on the fields of bacteriology and mycology, for which culture techniques are well established and for which culture is usually easy and fast, in opposition to viruses and protozoans.

I.2. The four tasks of the bacteriology and mycology laboratory.

Four general tasks can be recognized within clinical microbiology. The first objective is to answer the question: is there a pathogen present in the sample? This is called detection, whereby the presence of a microorganism will be checked.

The second objective is to specify the nature of the detected organism. This is called identification, whereby the organism will be specified towards a genus and a species name.

The third objective is antibiotic (or antifungal) susceptibility testing. Hereby the efficacy of different antibiotics (antifungals) to eradicate the bacteria or fungi (bactericidal activity) or to stop their replication (bacteriostatic activity) will be evaluated.

Finally there is the field of typing, whereby the relationship between different isolates is investigated. This leads towards essential information used to exclude outbreaks or investigate them to determine whether a central source or patient-to-patient transmission caused the outbreak.

It must be noticed that detection and identification are not always separated. For instance, in case selective media or species specific primers are used, detection and identification occur simultaneously when growth is observed on the selective medium or when a positive amplification signal is obtained. However, in most cases broad range media are used, whereafter the clinical microbiologist decides which of the colonies need further study and identification, or universal primers are used, for which amplification simply points to the presence of bacteria, whereafter additional hybridization, restriction digestion or specific amplification are needed for final identification.

This doctoral work is focused on identification of bacteria and fungi. The possibilities of detection, susceptibility testing and typing have been studied occasionally and will be dealt with when appropriate.

II. Aim of the research

The aim of the research was basically the development of PCR-based DNA-fingerprinting techniques for the identification of cultured bacteria and fungi in the routine clinical microbiology laboratory, and to evaluate their use.

The emphasis on PCR-based identification of cultured organisms may seem odd at first, since PCR has been considered from the very beginning as a very specific and sensitive alternative to culture-based detection and not as an approach to be used in combination with culture.

This approach is chosen because molecular techniques for detection of pathogens and for the determination of antibiotic resistance have several disadvantages and/or are too specialized.

Phenotypic identification has as limitations that - even for some of the major pathogenic species and certainly for less-frequently occurring or less well-known pathogens - it is frequently cumbersome and unreliable. Moreover, in the past decades a wide variety of bacteria traditionally thought to be simple components of the normal body flora have been implicated in infections, most frequently in aged, debilitated or immunocompromized patients, and several of these are difficult to identify with phenotypic techniques well.

III. Rationale for the development of culture-dependent PCR-based identification.

In order to provide with a rationale basis for the development of culture dependent PCR-based identification methods, it is necessary to first pinpoint the several problems and shortcomings that exist for both DNA/RNA-based detection and susceptibility testing of bacteria and fungi as well as for phenotypical identifications.

III.1. Nucleotide based detection and its shortcomings for routine use.

III.1.1. Principles of DNA-based detection.

In the field of detection the task is to reveal as rapidly, as accurately and as sensitively as possible the presence of a certain pathogen in the clinical sample. For this purpose molecular techniques appeared to be very interesting because they possess the possibility to start from low numbers of bacteria. Besides intensive research in non-profit organizations on PCR-based detection, also commercial companies have established research on this topic, because diagnostic tests for direct detection are marketable. Two sorts of molecular techniques are suitable for DNA-based detections.

III.1.1.1. DNA-hybridization techniques.

Hybridization tests are reactions whereby small synthetic manufactured oligonucleotides (probes) complement with a target region in the genome of the organism. The hybridization event then is visualized using a label on the oligonucleotide.

A well-known application is FISH (fluorescent *in situ* hybridization) (Bond and Banfield, 2001; Franks *et al.*, 1998). Since the sensitivity is strongly dependent on the amount of starting material, signal amplification techniques were developed to increase sensitivity. After hybridization, other oligonucleotides are added, coupling towards the target-oligo hybrid, constructing a complex structure resulting in a more intense signal: branched DNA (Nolte, 1998) and the detection system used in the Hybrid Capture[®] system (Pretorius *et al.*, 2002).

III.1.1.2. DNA-amplification techniques

These techniques have nucleic acids as target, which are exponentially amplified to a level notable through simple agarose gel electrophoresis and ethidiumbromide staining. The most

common and well-known technique is the Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987). PCR makes possible the exponential amplification of DNA regions in between the primer annealing sites, in a couple of hours. Diagnostic applications were described soon thereafter (Saiki *et al.*, 1988) and with the introduction of a thermostable DNA polymerase isolated from the bacterium *Thermus aquaticus* (the *Taq* DNA polymerase) (Saiki *et al.*, 1988b) PCR became widely used.

The PCR protocol involves cycles of heating the sample for denaturing, annealing of the primers, and elongation of the primers by a thermostable DNA polymerase. This 3 temperature cyclus is repeated approximately 30 times and gives an exponential doubling of the region between the primer annealing sites in every cycle starting from cycle 2. The final result is a high amount of duplicates of the target DNA, which can be visualized by gel electrophoresis and ethidiumbromide staining.

Other amplification protocols are: nucleic acid sequence based amplification (NASBA) (Compton, 1991), Ligase chain reaction (LCR) (Barany, 1991), Q beta amplification (Lizardi and Kramer, 1991), self-sustained sequence replication (3SR) (Fahy *et al.*, 1991) and strand displacement amplification (Walker *et al.*, 1992).

III.1.1.2.1 Species-specific amplification.

Several publications deal with the detection of a single species directly from the sample. For this purpose a wide variety of species-specific genes and primers have been used (Gynzbergen *et al.*, 1995). Different technologies have been established to gain time; e.g. faster cyclers (Bergeron *et al.*, 2000) or to gain sensitivity; e.g. immunocapture (Peng *et al.*, 2002). All this makes direct detection more promising, but the availability of these techniques is usually restricted to specialized groups (in particular difficult or slow growing species), the tests are expensive and technically complex or labor-intensive. All this makes that the cost of the test is, currently and probably still in the near future, not compensated by the gain of information and time.

III.1.1.2.2. Universal or broad-range amplifications.

When using primers complementary with conserved regions of a gene that is present in all or in most bacteria/fungi (e.g. 16S rRNA gene or 18S rRNA gene) it is possible to amplify this region whatever bacterium or fungus is present.

The regions used as target have to fulfill some major conditions: (i) to contain a constant sequence for all members, useful as primer binding site; (ii) to have interspecies variability in

the amplified region between the two primer-sites, so that this characteristic can be used to discriminate between species; (iii) to have a slow mutation rate so that its sequence is constant for a given species. This is mostly the case for genes coding for a compound essential in DNA replication, transcription and translation or for house-keeping genes.

Several regions in the chromosome can be used as a target for universal or broad-range amplifications. The best known for bacteria is the 16S rRNA gene (Wilson *et al.*, 1990) and 18S rRNA gene for eucaryotes like the fungi (Takashima and Nakase, 1999). Discrimination between the different organisms can be carried out using post-amplification species-specific hybridizations.

Other universal PCRs generate fingerprints, which are not suited for detection, because mixed fingerprints obtained by amplification of different organisms are not interpretable. Another disadvantage is that with lower sensitivity sometimes partial fingerprints are obtained, making interpretation more difficult.

DNA-fingerprinting PCRs are better suited for identification of cultured organisms and will be discussed later (III.3).

III.1.2. Problems of PCR-based detection.

After almost two decades of worldwide research, the use of molecular detection systems in clinical microbiology remains restricted nearly exclusively to the field of virology. Several factors make that DNA-technology for the detection of bacteria will remain applicable only for slowly growing bacteria like *Mycobacterium tuberculosis* and *C. trachomatis*.

The applicability in the fields of bacteriology and mycology is totally different than for virology (Table 1).

III.1.2.1. Culture of bacteria and fungi is easy, fast and flexible.

Most of the pathogenic bacteria can be cultured on simple media after overnight incubation.

When using an appropriate medium, only 24 hours of incubation are needed, in contrast to virus culture. DNA/RNA-based detection consists of several steps including efficient

DNA/RNA-extraction, an amplification reaction and the detection of the PCR products.

Compared to bacterial culture, the gain in time is minimal, and the process is much more

laborious. Culture is also more flexible because it is impractical to start the DNA-

amplification process for one or a few samples, while inoculation of a culture medium can be done at any time for a single sample.

III.2.2. Culture provides a semi-quantitative estimate.

The obtained culture delivers living cells that can be used for identification, but also gives additional information. It gives an indication towards the quantification of the organism in the sample, and an overview of the different species in mixed samples. For example: throat and faeces are routine clinical samples containing a wide variety of bacteria.

To obtain the same amount of information using DNA-technology quantitative amplification of very different bacteria should be carried out.

III.1.2.3. A wide variety of bacterial pathogens need to be covered.

The number of bacterial organisms able to cause infection is so diverse that setting up a direct detection technique able to cover whatever pathogen in a clinical sample needs such a large collection of species-specific primers or probes that it is hardly possible to achieve. DNA-arrays seem to offer a solution at first sight, but the simultaneous amplification of DNA of different bacteria can quite often generate noise instead of signal and competition between the different targets present may inhibit the amplification of several species.

III.1.2.4. Antibiotic susceptibility testing will remain culture-based.

During the last 50 years the treatment of bacterial infections changed dramatically due to the introduction of antibiotics, starting with penicillin by Fleming in 1928 (Chain *et al.*, 1993). But after half a century of administering antibiotics to patients, resistance has emerged in almost every possible pathogen. The pressure of antibiotic treatment is selecting those cells that have obtained resistance against the antibiotic. Antibiotic resistance can be caused by a variety of mechanisms: (i) the presence of an enzyme that inactivates the antimicrobial agent (destruction); (ii) the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent (bypass); (iii) modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent (target); (iv) reduced uptake of the antimicrobial agent (permeability); (v) active efflux of the antimicrobial agent (permeability); and (vi) overexpression of the target of the antimicrobial agent. In addition it is also possible that resistance is caused by still unknown mechanisms.

The clinical microbiologist should be able to select those antibiotics best suited for antibiotic/antifungal treatment and therefore antibiotic susceptibility testing is primordial in clinical microbiology. The change of culture-based susceptibility testing towards genotypic testing is however not obvious, because of the high variety of genes involved in antibiotic

resistance and the limited knowledge of the complex processes involved. On the other hand, routinely used culture-based antibiotic susceptibility tests are efficient for the determination of the susceptibility without the need for knowledge of which mechanism is responsible for the resistance.

The best known method is the Kirby-Bauer Disk Diffusion method (Figure 1). This method enables the simultaneous detection of the susceptibility of the strain towards a variety of different antibiotics, which may be of very different nature and for which resistance may be caused by very different genes and mechanisms. After incubation the susceptibility of the strain for every antibiotic separately can be noted and in some cases also synergetic and/or antagonistic effects can be observed.

Figure 1: Kirby-Bauer Disk Diffusion testing.



Legend: Bacteria are cultured in presence of a collection of paperdisks (each containing a different antibiotic). If the strain is resistant to a specific antibiotic, no inhibition of growth is seen around that disk.

Depending on the mechanism of resistance, different levels of antibiotic resistance are possible. A strain with a low level of resistance can still be treated with the antibiotic if the dose is high enough. A precise indication of the dose of the antibiotic still effective in the treatment of a certain bacterium can be obtained using the E-test. The E-test makes it possible to determine the Minimal Inhibitory Concentration (MIC) values of the strain in a simple and fast manner (Figure 2).

Figure 2: MIC-determination using E-test



Legend: Bacteria are cultured in presence of a paper strip containing a descending concentration of antibiotic (E-test). The MIC value is detected by reading the concentration on the strip where the inhibitory zone intersects the strip.

These culture-based methods usually give a fast and (semi-) quantitative answer about the efficiency of certain antibiotics. The replacement of culture-based testing with genomic testing faces several problems. The total number of genes responsible for resistance is high. This results in a very large amount of genes to be screened at once, leading towards a high amount of different PCRs and laborious post-amplification manipulations with a variety of probes or sequencing reactions.

Besides the known genes and their mutations, there are probably many unknown mechanisms, resistance genes and mutations. Other resistance mechanisms such as hyperproduction or higher efflux are difficult to monitor by genotypic testing and require a quantitative RNA-based approach.

In summary, molecular techniques are very useful for fundamental research of resistance mechanisms, but are difficult to use for antibiotic resistance testing in a routine microbiology laboratory. The recent introduction of microarrays makes it possible to test a large number of probes (Westin *et al.*, 2001). However, the numerous amplification reactions needed to screen all those genes remain the limiting factor.

Even when the problem of the numerous amplifications can be solved, and microarrays with the probes to all known resistance genes are available, there is still the lack of information coming from unknown genes. A final problem is that resistance in several cases depends on the level of expression of certain genes (Livermore, 1987; Liu *et al.*, 1992), which requires quantitative RNA amplifications.

Because of those disadvantages, resistance screening is restricted to the search for some standard resistant markers, like the detection of the *mecA* gene for methicillin resistance in *Staphylococcus* species (Maes *et al.*, 2002) and the *van*-genes for vancomycin resistance in *Enterococcus* species (Dutka-Malen *et al.*, 1995; Lu *et al.*, 2001). Because these genes are known as the predominant cause of the resistance in that kind of organisms, these tests have high predictive values. An additional advantage of the use of the *mec* and *van*-PCRs is that *in vitro* detection of resistance is sometimes difficult to interpret in those species.

Table 1: Basic differences between Virology and Bacteriology/Mycology

	Virology	Bacteriology / Mycology
Culture	Difficult and time-consuming	Easy, fast and flexible*
Diversity of organisms	Limited number of clinically important viruses	Wide diversity of bacteria and fungi able to infect humans.
Therapy control	Limited number of antiviral drugs	Wide range of antibiotics Growing range of antifungals
	Good knowledge of mutations causing resistance	Already numerous mutations known, but still several other regulation mechanisms are unknown or can not be detected genotypically

* Most important exceptions: *Mycobacteria* and *Chlamydia*.

III.1.2.5. Other problems of DNA-based detection: sensitivity and contamination.

The DNA-based methods are always referred to as highly sensitive, but some clinical samples contain low amounts of bacteria in a rather large volume, making efficient DNA-extraction tedious. For example: Stratton (1994) stated that in some cases of sepsis up to 50 ml of blood should be cultured to detect the causative organism. Extracting DNA of so few bacterial organisms amid a high amount of human DNA seems an impossible task. Still automated blood culture systems have no problem detecting such low amounts of living bacteria, mostly within one day.

On the other hand, high sensitivity increases the chance for false positives, because of low amounts of contaminating bacterial DNA present in the samples. It has been shown that blood of healthy people can contain bacterial ribosomal DNA, without any clinical significance (Nikkari *et al.*, 2001).

Another source of contamination is previously amplified material, which can be present because of the high amounts of material obtained by amplification and because of the different post-amplification manipulations. Therefore, good monitoring of the overall process is needed (Noordhoek *et al.*, 1994). And finally, there is the problem of sample collecting material and PCR reagents that are not totally free of bacterial DNA (Millar *et al.*, 2002). So direct detection PCR needs setting up a specialized facility and the availability of laboratory and sample collection material completely free of DNA.

All these obstacles lead to the conclusion that PCR-based direct detection of most bacterial (and fungal) pathogens directly from clinical samples is too tedious and too laborious without much gain in time and even with less information than can be obtained with culture.

Only in some cases direct detection could be beneficial, like for slowly-growing pathogens or species for which there are no effective culture media. The most important pathogens showing those characteristics are *Mycobacterium tuberculosis*, which is a slow grower (up to three weeks for a positive culture) and *Chlamydia trachomatis* and *Mycoplasma pneumoniae*, pathogens routinely detected by serology instead of culture. Other targets are the unculturable pathogens (Relman, 1993) with as examples *Tropheryma whippelii* (Relman *et al.*, 1992) causing Whipple's disease and the hardly culturable pathogens, like *Bartonella henselae* (Koehler *et al.*, 1994), the infectious agent causing cat-scratch disease, and others like spirochetes, *Legionella* and *Campylobacter*.

This is also the reason why commercial companies only for some of those pathogens successfully introduced direct detection kits.

III.1.2.6. Need for grown material for epidemiology control.

The change towards molecular detection has an important disadvantage that no cultivated material is present anymore, which is needed for epidemiology control. The demands for typing comes mostly afterwards (after several isolates of the same species are seen) and when using only molecular techniques for detection, this can result in the unavailability of living material.

III.1.3. Conclusion: general problems of DNA-based detection.

It can be stated that PCR-based detection of most bacterial pathogens is not competitive with culture-based detection because of higher cost, laboriousness and lower flexibility, for no gain in speed and sensitivity. Even if it were possible to develop practically applicable and

affordable PCR-based detection of all bacterial pathogens, it will remain impossible to replace culture-based susceptibility testing by molecular techniques. Therefore, culture will remain unavoidable, even in the imaginary case that PCR-based detection would be possible. In what follows, we will focus on the development of molecular-based identification of cultured pathogens.

III.2. Phenotypic identification of cultured organisms and its shortcomings.

III.2.1. Introduction: the importance of identification.

The isolation of a microorganism from a clinical sample can have several meanings. Some species are commonly encountered in some parts of the body as commensals and can even have beneficial properties, like *Lactobacillus* in the vagina (Cadieux *et al.*, 2002) and gut, leading to applications as probiotics (Gibson and Robertfroid, 1995). However, several species are known as pathogenic and are the cause of infections. This makes it important to identify an isolate as specifically as possible, to make a decision with regard to therapy and prognosis. The better the knowledge of the infectious organism, the better the therapy can be directed towards that organism.

Due to presence of a higher number of immunocompromised patients, and due to the extensive use of antibiotics, an increasing number of bacterial species is causing diseases in humans. This renders the identification based on biochemical properties more difficult.

For yeasts this trend is even more distinct: 15 years ago 80% of the invasive yeast infections were due to *C. albicans*. Nowadays, however, already half of the infections are caused by non-*C. albicans* species (De Pauw, 2002; Nguyen *et al.*, 1996), a phenomenon that is presumably related to the extensive prophylactic use of fluconazole (Rex and Sobel, 2000). There are also big differences between the natural antifungal resistance patterns of those species, and several non-*C. albicans* species show increased resistance for antifungals (Vasquez *et al.*, 2001b).

Moreover, there are differences in pathogenicity between the *Candida* species: *Candida albicans* is known as the most virulent species, but also *C. tropicalis* and other non-*C. albicans* species can be virulent, whereby others are known to be less virulent like *C. parapsilosis* (Wingard, 1995; Moran *et al.*, 2002).

All of this makes that accurate identification of yeasts also provides guidelines for therapy and that rapid identification may save time in choosing the optimal therapy.

III.2.2. Overview of different identification technologies.

Identification aims to classify an unknown isolate into a group of bacteria with shared phenotypic and genotypic characteristics (a taxon). This can be achieved in several ways.

III.2.2.1. Morphological characteristics

The morphological characteristics used for the identification of bacteria and yeasts, can be subdivided in two classes: (i) macroscopic (colony) morphology, consisting of properties, noticeable on the culture plate, like the color, shape or scent of the colony and (ii) the microscopic (cellular) morphology or properties to be noticed by microscopic investigation, like the shape of the bacteria (coccus, bacillus or spirillum) and yeasts and Gram stain: Gram positive or Gram negative bacteria.

A disadvantage is the low discriminatory power. General groups can be distinguished but related species have mostly common morphological characteristics. Also sometimes misinterpretations can be made by Gram positives known to destain (e.g. *Clostridium tertium* (Steyaert *et al.*, 1999)).

III.2.2.2. Biochemical properties

When testing biochemical characteristics, the organism is checked for its capability to use certain carbon sources, or to perform certain enzymatic reactions. Biochemical testing is more informative than morphological investigation, resulting in a better classification and identification.

Biochemical testing originally made use of single tubes or media scoring one or a few biochemical characteristics. Since the 1970s commercial multi-well galleries were developed, for example Api (BioMérieux, Marcy-l'Etoile, France); Crystal (Becton Dickinson, Sparks, Md.) and Auxacolor (Sanofi Pasteur, Marnes la Coquette, France).

These are micromethods employing a collection of different biochemical tests, one per microwell that already contains the chemical substrates. After addition of a suspension of the organism and incubation, the biochemical profile of the strain is obtained. The Api strip generates a 7-digit code based upon the reactions, which can be compared to the database of the system, containing all codes corresponding to the species included. The digital code can then be converted to a bacterial identification.

There are however disadvantages: (i) many new species are continuously described (Euzéby, 1997) and several of these need other biochemical tests to be discriminated from already established species;

(ii) Biochemical testing has the disadvantage that biochemical properties can be unstable at times and vary with environmental changes, like media, temperature, pH, age of culture, aerobic or anaerobic cultivation, etc. (Rossello-Mora and Amann, 2001);

(iii) Biochemical profiles of different species can be identical, such that identification of those species is difficult by biochemical testing. This is e.g. the case for *Pasteurella* (Hamilton-Miller, 1993) and *Haemophilus* species (Hamilton-Millar and Shah, 1996);

(iv) The tests are read visually and need interpretation so that weak reactions can be misinterpreted, leading to misidentification.

Regardless those disadvantages, biochemical testing is mostly still effective (O'Hara *et al.*, 1992) and very commonly used. New, automated biochemical test systems like Vitek (BioMérieux) and Phoenix (Becton Dickinson), which rely on sophisticated technology and on automated reading, interpretation algorithms of the color-reactions and growth curves, can eliminate some of the disadvantages.

The most automated systems have a panel of different reactions for identification and antibiotic resistance testing at once, and have very promising first evaluations (White *et al.*, 2001; Williams *et al.*, 2001).

III.2.2.3. Antigenic testing

Identification can be obtained by the strains' affinity towards a wide collection of different antisera or antigens. These tests are not always sufficiently discriminative because cross-reactions between members of the same genus are common (Houpikian *et al.*, 2002). Another drawback is the need for a wide variety of different antisera and antigens. Commercial agglutination tests are available for a limited number of species, for example: *Neisseria meningitidis* (Wellcogen *N. meningitidis*; Murex, Darford, United Kingdom), *Streptococcus pneumoniae* (Streptococcal grouping kit; Oxoid, Basingstoke, United Kingdom) and *Staphylococcus aureus* (Pastorex Staph-plus; Biorad, Hercules, Ca.).

III.2.2.4. Chemotaxonomic methods

With developments in all fields of molecular biology, specialized techniques were developed for bacterial identification like (i) SDS-PAGE analysis of whole-cell proteins (Pot *et al.*, 1994; Premaraj *et al.*, 1999); (ii) gas chromatography of fatty acids (Sasser, 1990; Lehtonen *et al.*, 1996), (iii) high-performance liquid chromatography of mycolic acids (Butler *et al.*, 1986; Butler and Guthertz, 2001), the latter used for identification of mycobacteria; and (iv) spectra of the overall molecular composition of the organism, using the Raman spectroscopy (Maquelin *et al.*, 2000); (v) multivariate analysis of proton magnetic resonance spectroscopic data (Bourne *et al.*, 2001); (vi) immunomagnetic separation combined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy (Madonna *et al.*, 2001); and (vii) Fourier-transform infrared (FT-IR) spectroscopy (Oberreuter *et al.*, 2002; Wenning *et al.*, 2002).

The discriminatory power of these techniques is much higher but the techniques are also demanding for culture standardization and specialized equipment and seem not to be suited for routine applications. The most recent ones, the last four, seem very promising but their experience concerning discriminatory capacity and liability is far from sufficient at this moment.

III.2.3. Conclusion: general problems of phenotypic identification.

Phenotypical methods are based on the investigation of the genomic material in an indirect way, as it is expressed in cell-structure, enzymatic activity, etc. All these characteristics are dependant on growth conditions, which demands for very standardized test protocols. Except for the complex chemotaxonomic methods, which demand for very strict standardization to correlate the results, the discrimination power is often too low. For antigenic testing a variety of different antigens and antisera are required and this restricts the applicability of the method.

III.3. PCR-based DNA-fingerprinting.

III.3.1. Introduction.

DNA-based techniques have as an advantage that the genomic material of an organism is investigated in a direct way, independent of culture conditions.

In chapter III.1.1.2 DNA-amplification techniques are explained. The disadvantages for hybridization and species specific amplification for detection, are also disadvantages for DNA-based identifications with these techniques. An alternative is amplification with universal PCRs and post-amplification hybridization, and most likely by immobilization of the probes on a cellulose strip as in the Line Probe Assay (Innogenetics, Ghent, Belgium) (Tortoli *et al.*, 2001) or on a microarray (Westin *et al.*, 2001), enabling the screening for a large variety of organisms at once. However, the problem remains that one can only identify those species included in the test. Universal amplification can be carried out using several regions in the chromosome for which the best known for bacteria is the 16S rRNA gene (Wilson *et al.*, 1990). Other regions are *gyrB* (Yamamoto and Harayama, 1995; Kasai *et al.*, 1998), the spacer region between 16S and 23S (Gürtler and Stanisch, 1996; Jensen *et al.*, 1993; Song *et al.*, 2000), the tRNA-spacer (Welsh and McClelland, 1991), the *hsp65* gene (Telenti *et al.*, 1993), the *rpoB* gene (Drancourt and Raoult, 2002) and *rep* (Gevers *et al.*, 2001).

For fungi the 18S rRNA (Takashima and Nakase, 1999) and the internally transcribed spacer regions - ITS1, in between 18S and 5.8S rRNA genes (Chen *et al.*, 2001.), and ITS2, in between 5.8S and 28S rRNA genes (Turenne *et al.*, 1999, Chen *et al.*, 2000) - can be used.

After universal amplification, analysis of the amplification product is needed to catalogue the unknown isolate to assign it to an established group of strains (= taxon, usually a species). The maximum information is obtained by total sequence analysis of the fragment. However, this technique remains, even with the newer systems, still expensive and laborious, making it impractical and too slow for routine identification of isolates. Several other methods are applied to investigate fragments for sequence diversity, making the process of identification either cheaper, less-laborious and/or faster. If the obtained amplification products of different species have different sizes, determination of the fragment lengths leads to identification (Jensen *et al.*, 1993; Welsh and McClelland, 1991). If the fragment has always the same length but the sequence is variable, sequence dependent electrophoresis can reveal differences. Sequence dependent systems are single-stranded conformational polymorphism

(SSCP) (Moore *et al.*, 1992), denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993), temperature gradient gel electrophoresis (TGGE) (Heuer and Smalla, 1997) and temporal temperature gradient gel electrophoresis (TTGE) (Vasquez *et al.*, 2001).

Sequence differences can also be revealed by restriction analysis, because mutations can lead to the appearance or disappearance of restriction sites, changing the restriction pattern (e.g. ARDRA, Vaneechoutte *et al.*, 1992).

A recent DNA-fingerprinting technique is fragmentation by uracil-DNA-glycosylase of a 16S rRNA sequence, amplified in the presence of dUTP instead of dTTP, creating T-ending fragments, and mass-spectrometry analysis with matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) (von Wintzingerode *et al.*, 2002).

III.3.2. Advantages of the use of DNA-based fingerprinting for identification.

PCR-based fingerprinting as an identification tool has several advantages. The technique uses DNA as target for the amplification, so that the identification of the strain is culture-condition independent.

The identification is based on the comparison of the obtained fingerprint with a library of fingerprints. This library can be updated whenever new species are encountered. Another advantage of such libraries is that interlaboratory exchange is possible, such that information gathered in different laboratories can be compared and stored in a mutually accessible, common library.

As with every bacterial identification technique, there are some drawbacks.

First, universal primers will amplify every organism present. In mixed samples this can lead towards non-useful fingerprints in cases where the individual fingerprint consists of several bands (e.g. ARDRA, tRNA-PCR). When only one band is expected per fingerprint (e.g. ITS2-PCR) the approach could be used directly on mixed samples.

The differences between closely related species can be sometimes rather small. Sometimes minor, but reproducible and significant interspecies differences can be revealed by performing electrophoresis on high resolution systems like capillaries or sequencing gels, rather than on agarose gels.

IV. Development, update and evaluation of PCR-based DNA-fingerprinting techniques for identification of cultured bacteria and fungi.

IV.1. Introduction.

During this doctoral work, we focused on PCR-based fingerprinting techniques since these can be applied for most bacteria and fungi, using a single set of primers and since they are applicable in a routine setting due to their technical simplicity.

Three different techniques have either been developed or updated and evaluated.

At first there is ARDRA (amplified ribosomal DNA restriction analysis), where the polymorphism is obtained by restriction analysis of the amplicon, i.e. the amplified 16S rRNA gene.

Secondly there are two spacer region amplification techniques that result in a fingerprint by amplification without post-amplification manipulation.

tRNA-PCR amplifies the spacer region in between tRNA genes and is an identification system for bacteria, whereas ITS2-PCR, which amplifies the spacer region in between the 5.8 S and 28 S rRNA genes, is an identification system for yeasts.

The characteristics of these three techniques are summarized in Table 2.

Table 2: Fingerprinting PCRs of interest.

	ARDRA	tRNA-PCR	ITS2-PCR
Universal target	16S rRNA gene	tRNA spacer regions	Ribosomal spacer region
Post-PCR manipulation	Restriction digestion Agarose gel electrophoresis	Capillary electrophoresis	Capillary electrophoresis
Result of electrophoresis	Multiple band fingerprint	Multiple band fingerprint	Single band with species specific length
Best suited for	<i>Mycobacterium</i> <i>Corynebacterium</i> <i>Clostridium</i> <i>Acinetobacter</i>	Bacteria (excl. <i>Mycobacterium</i> , <i>Corynebacterium</i>)	Yeast (Molds)*

* Currently ITS2-PCR is being updated for identification of molds, using an additional restriction digestion step. In this respect, ITS2-PCR + restriction is somewhat comparable with ARDRA

IV.2. An overview of the techniques used.

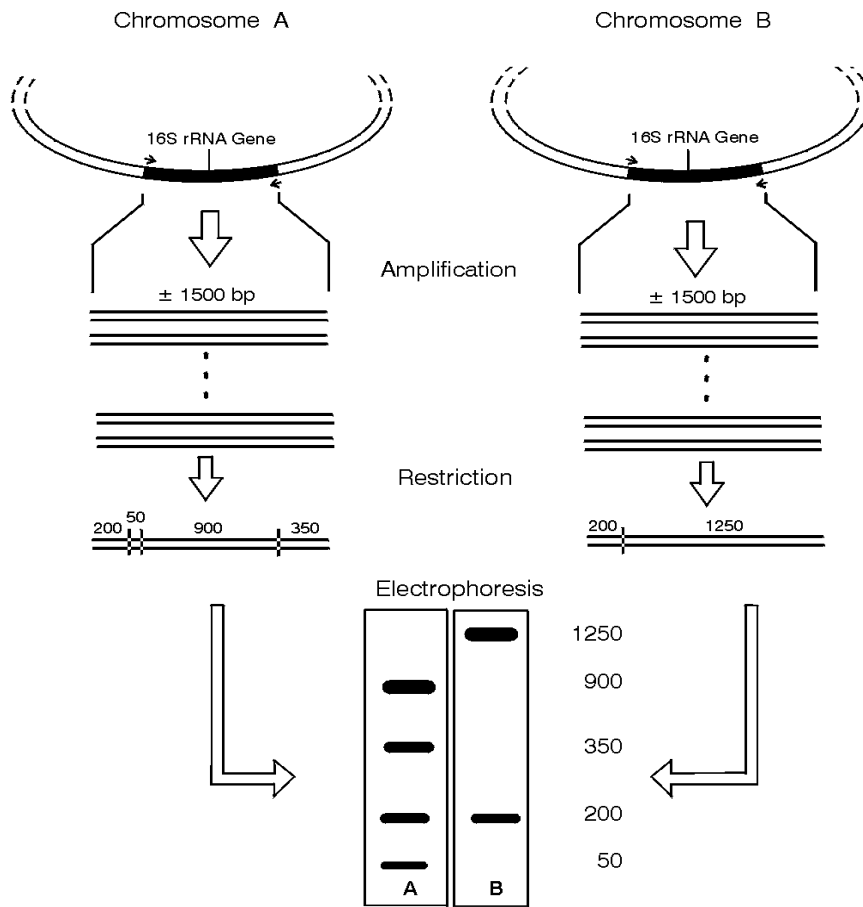
IV.2.1. Amplified ribosomal DNA restriction analysis (ARDRA).

ARDRA was first applied already a decade ago (Vilgalys and Hester, 1990, Jayarao *et al.*, 1992, Vaneechoutte *et al.*, 1992), and has as target different parts of the rRNA cistron, although the 16S rRNA gene (approximately 1500 basepairs) is used in most studies. The 16S rRNA gene is found in all bacteria and accumulates mutations at a slow, constant rate, and can therefore be used as a molecular clock (Woese *et al.*, 1987). However, exceptions were encountered for *Aeromonas* (Sneath, 1993; Graf, 1999), where mosaic 16S rRNA-genes occur, possibly as a result of homologous recombination between species. Some parts of the 16S rRNA gene are highly variable and contain signature sequences unique for a species. The variability is genus dependent. It is high in for example *Mycobacterium*, *Clostridium* and *Acinetobacter*, but low in other genera like *Enterococcus* and *Staphylococcus* (Vaneechoutte and Heyndrickx, 2001).

ARDRA consists of the amplification of the 16S rRNA gene by primers directed towards the 5' and 3' regions of the gene, which are conserved throughout the kingdom bacteria. The amplicon is subjected to restriction digestions with one to four different restriction enzymes, each resulting in a mixture of restriction fragments with different lengths. These fragments are separated by agarose gel electrophoresis (Fig. 3). The obtained fingerprint is compared to a photograph containing previously observed fingerprints with that enzyme for the known species of the genus. To attain a higher discriminatory power, the results of several (mostly three) different restriction reactions are combined. This results in a 1 to 4-digit (depending on the amount of enzymes used, mostly it is a 3-digit) code, which leads towards identification. A disadvantage of the technique is the limitation of the range of organisms restricted to members of the same genus, because the choice of restriction enzymes is genus dependent. Most of its limitations are related to problems in general with the 16S rRNA gene (see Bacterial Systematics, page 67 and followings).

ARDRA is, however, a fast, easy and cheap identification technique for members of different genera, which is illustrated by publications on species differentiation of *Corynebacterium* (Vaneechoutte *et al.*, 1996) and *Acinetobacter* (Dijkshoorn *et al.*, 1998). A complete overview can be found in Vaneechoutte and Heyndrickx (2001).

Figure 3: Principle of ARDRA



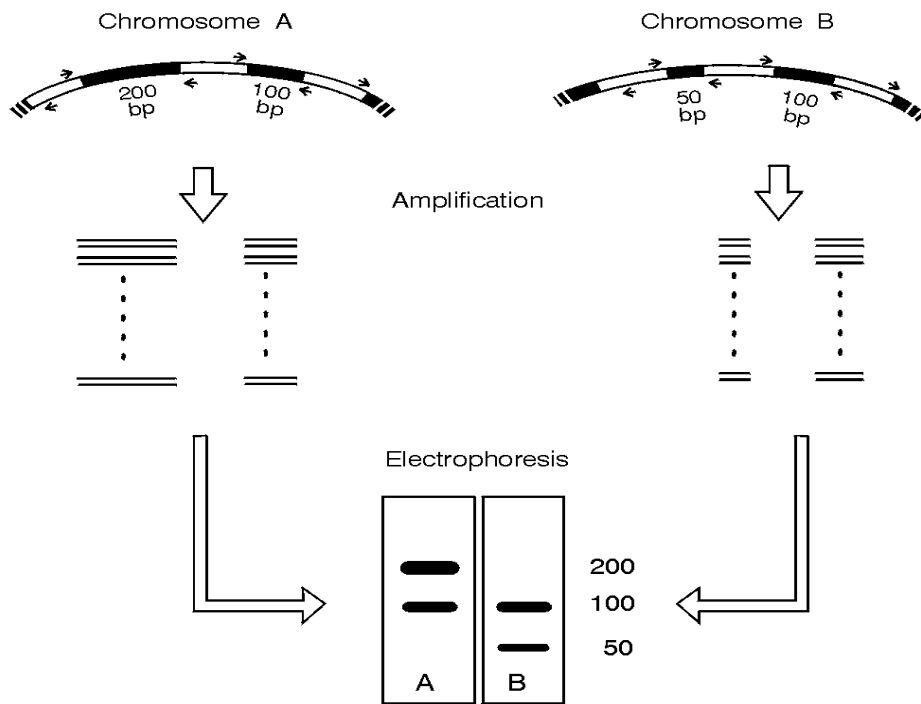
→ : universally applicable primers, complementary to strongly conserved regions at the edge of the 16S rRNA-gene (rDNA).

IV.2.2. tRNA-intergenic spacer length polymorphism analysis (tRNA-PCR).

tRNA molecules are the amino-acid transporter molecules in the translation of mRNA to proteins. Although 20 different tRNA genes are present in a varying number of alleles in the genome, their structure is well conserved in some regions. The sequences at the outer parts of the gene are identical for most tRNA genes in most bacteria, such that consensus, universal primers can be developed (Welsh & McClelland, 1991). In tRNA-PCR the primers are outwardly directed such that the intergenic spacers between subsequent tRNA genes are amplified. These inter-tRNA gene spacers are different in length, so that amplification results in a fingerprint consisting of multiple bands (Figure 4). Moreover, the obtained fingerprints are mostly species specific, although intraspecific variability exists and some species cannot be distinguished from each other.

Since the differences in length are sometimes very minor, high resolution electrophoresis must be used. This can be obtained by adding a fluorescent label to one of the primers and analysing the obtained fragments on a capillary electrophoresis system, in this case the ABI Prism 310 (Applied Biosystems, Foster City, Ca.) is used, which provides with a one base pair resolution. The final result is a digital fingerprint. All those fingerprints are collected in a library. If an unknown strain is tested, the obtained fingerprint can be compared to all previously obtained fingerprints, resulting in an identification. A software program Basehopper, especially designed for this application (Ing. Paul Baele, Ghent University), simplifies this comparison.

Figure 4: Principle of tRNA-PCR



→ consensus primer, outwardly directed

□ tRNA gene

■ interrepeat spacer

IV.2.3. ITS2 spacer length polymorphism analysis (ITS2-PCR).

The internally transcribed spacer region 2 is the spacer region in between the 5.8S rRNA and 28S rRNA genes of fungi, and thus part of the ribosomal cistron. The ribosomal cistron has a slow mutation rate as mentioned for the 16S rRNA gene of bacteria.

The primers chosen are complementary with conserved regions lying at the 3' end of the 5.8S rRNA gene and the 5' end of the 28S rRNA gene. The primer pair is universal for all fungi. Discrimination between most yeasts is possible due to the length differences of this spacer region, which are constant for a given species but differ from one species to another. The obtained fragment length leads towards identification (Turenne *et al.*, 1999).

Because the fingerprint consists of only one fragment, the discriminatory power is lower than for multiple fragment fingerprints. A precise determination of the exact size of the fragment is needed for optimal identification. This can be obtained by labelling one primer with a fluorescent label, and analysis of the fingerprint on a capillary electrophoresis system (ABI Prism 310). The digital fingerprints of different species can be collected in a library, and comparison of the fingerprint of an unknown strain to the library results in identification. This can be done using the Basehopper software.

IV.2.4. DNA-sequencing

Sequencing is the technique providing the maximum information for an amplified product. Some laboratories are using this technique for reliable identification of unknown bacterial strains (Drancourt *et al.*, 2000; Hill *et al.*, 2002; Takashima and Nakase, 1999).

First, an amplification of the 16S rRNA gene or ITS2 region with universal primers is carried out. After purification of this amplification product, a sequencing reaction is done. The obtained dideoxy terminated fragments are separated and analysed on sequence devices (gel- or capillary based). Assembling of different fragments and other analysis on the sequence data is carried out using software programs. The obtained sequence can then be compared to all known sequences in Genbank using Blast 2.0 (National Center for Biotechnology Information, Bethesda, Md. [<http://www3.ncbi.nlm.nih.gov/BLAST/>]). The program returns a list of sequences sorted according to descending similarity.

Although the technique is resulting in the maximum on information, there are several disadvantages.

The sequences in Genbank are not always of high quality (Turenne *et al.*, 2001), which can be solved with the use of the commercial databases like Microseq (Applied Biosystems) (Patel *et al.*, 2000) or Ridom (Ribosomal Differentiation of Medical Microorganisms), which are databases containing confirmed and validated sequences. This makes the process however more expensive.

A second problem is interpretation of sequence similarities. Sometimes, members of the same species do not have identical sequences (Harrington and On, 1999). And to the opposite, members of different species may have almost identical 16S rRNA gene sequences:

Aeromonas (Graf, 1999), *Bacillus*, *Staphylococcus*.

Also, ambiguities can be present due to direct sequencing starting from a strain carrying different 16S rRNA-alleles (Gürthler, 1999; Reisch *et al.*, 1998).

Besides these disadvantages, there are still some practical problems: (i) sequencing remains technically demanding and requires specialized material (consumables, machinery) and experienced personnel; (ii) the complete process including analysis is rather laborious, and (iii) all this makes that it is more expensive than other techniques.

Conclusion: Sequencing is a highly discriminatory technique, however, not applicable for routine identification. It is a very important tool for the confirmation of presumed identifications or to identify organisms unidentifiable by other techniques.

IV.3. Studies.

Our studies aimed to introduce molecular identification techniques in our laboratory as an alternative for phenotypical identification techniques. Therefore, the techniques tRNA-PCR and ITS2-PCR were introduced and ARDRA (for mycobacteria) was updated. All techniques were evaluated for their application as possible useful routine identification techniques.

If in some cases identification was not possible or questionable, sequencing of either the 16S rRNA gene or the ITS2 region, followed by BLAST to Genbank, resulted in an additional and mostly reliable identification. The obtained information was used for updating and confirming the databases of the used techniques.

IV.3.1. The update and evaluation of ARDRA for *Mycobacterium* species identification.

De Baere, T., R. de Mendonca, G. Claeys, G. Verschraegen, Mijs, W., R. Verhelst, S. Rottiers, L. Van Simaey, C. De Ganck, and M. Vaneechoutte. 2002. Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of cultured mycobacteria in a diagnostic laboratory. *BMC Microbiology* **2**:4.

IV.3.2. The introduction of tRNA-PCR for bacterial species identification, and the study of interlaboratory exchangeability of the fingerprints

Baele, M., V. Storms, F. Haesebrouck, L.A. Devriese, M. Gillis, G. Verschraegen, T. De Baere, and M. Vaneechoutte. 2001. Application and evaluation of the interlaboratory reproducibility of tRNA intergenic length polymorphism analysis (tDNA-PCR) for identification of species of the genus *Streptococcus*. *J. Clin. Microbiol.* **39**:1436-1442.

IV.3.3 The evaluation of ITS2-PCR for routine identification of yeasts.

De Baere, T., G. Claeys, D. Swinne, C. Massonet, G. Verschraegen, A. Muylaert, and M. Vaneechoutte. 2002. Identification of cultured isolates of clinically important yeast species using fluorescent fragment length analysis of the amplified internally transcribed rRNA spacer 2 region (ITS2). *BMC Microbiology* **2**:21.

Methodology article

Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of cultured mycobacteria in a diagnostic laboratory

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Abstract

Background: The development of DNA amplification for the direct detection of *M. tuberculosis* from clinical samples has been a major goal of clinical microbiology during the last ten years. However, the limited sensitivity of most DNA amplification techniques restricts their use to smear positive samples. On the other hand, the development of automated liquid culture has increased the speed and sensitivity of cultivation of mycobacteria. We have opted to combine automated culture with rapid genotypic identification (ARDRA: amplified rDNA restriction analysis) for the detection resp. identification of all mycobacterial species at once, instead of attempting direct PCR based detection from clinical samples of *M. tuberculosis* only.

Results: During 1998–2000 a total of approx. 3500 clinical samples was screened for the presence of *M. tuberculosis*. Of the 151 culture positive samples, 61 were *M. tuberculosis* culture positive. Of the 30 smear positive samples, 26 were *M. tuberculosis* positive. All but three of these 151 mycobacterial isolates could be identified with ARDRA within on average 36 hours. The three isolates that could not be identified belonged to rare species not yet included in our ARDRA fingerprint library or were isolates with an aberrant pattern.

Conclusions: In our hands, automated culture in combination with ARDRA provides with accurate, practically applicable, wide range identification of mycobacterial species. The existing identification library covers most species, and can be easily updated when new species are studied or described. The drawback is that ARDRA is culture-dependent, since automated culture of *M. tuberculosis* takes on average 16.7 days (range 6 to 29 days). However, culture is needed after all to assess the antibiotic susceptibility of the strains.

Background

Rapid and accurate detection, identification and susceptibility testing of mycobacteria remains important i) be-

cause the overall incidence of tuberculosis is increasing, also due to the HIV pandemic [1], ii) because of the increasing resistance to antituberculous agents [2] and iii)

because an increasing number of mycobacterial species are being recognized as potentially pathogenic [3]. The importance of *M. avium* infection has increased in HIV patients [4], and clinical infections have been described with species like *M. heidelbergense* [5], *M. conspicuum* [6], *M. branderi* [7] and *M. interjectum* [8,9], which have been recognized only recently.

Current DNA amplification based diagnostic tests are expensive, have limited sensitivity, are usually restricted to the detection of *M. tuberculosis* only and provide no or limited information on susceptibility (e.g. rifampicin only: RifTB LiPA, Innogenetics, Zwijnaarde, Belgium). Therefore, the need for culture has not been circumvented. The CDC decided to restrict the use of genotypic tests to confirmation of smear positive samples, so that they cannot be used to test the large number of specimens processed for mycobacterial detection every year in an average laboratory [10]. Only recently, the enhanced AMTD (GenProbe, San Diego, CA) gained FDA approval for direct detection of *M. tuberculosis* from smear-negative samples, but several problems are reported [e.g. [11], and the high costs keep restricting its use.

Here, we present our findings with the use of ARDRA [12] for the identification of *Mycobacterium* species. The method consists of amplification of the 16S rRNA gene (rDNA) and subsequent restriction digestion of the amplicon. The restriction patterns obtained with different restriction enzymes and combination of these patterns into a restriction profile was shown to enable identification of most clinically important mycobacteria by comparison of the obtained profiles with a library of ARDRA profiles obtained for reference strains of different species [12]. This PCR-RFLP analysis of the 16S rRNA gene, was published almost simultaneously with the more widely used technique (known as PRA), which is based on the amplification of the *hsp65* gene [13–15].

Results

The initial study describing the applicability of ARDRA for the identification of mycobacteria [12] used universal bacterial primers. However, this sometimes resulted in the false positive amplification from decontaminated samples of organisms other than mycobacteria. Therefore primers were developed, aimed at more specific amplification of mycobacteria. During the three year evaluation period, of which the results are reported here, amplification of nonmycobacterial organisms occurred in two cases. These organisms, namely *Corynebacterium glutamicum* and *Actinomyces odontolyticum*, stained acid fast on direct smear and are relatives of the mycobacteria.

The restriction patterns obtained with the enzymes *HhaI* (isoschizomer of *CfoI*), *MboI* and *RsaI* for the different spe-

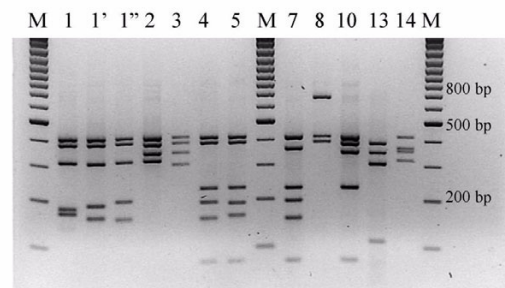


Figure 1

HhaI (*CfoI*) restriction patterns of amplified mycobacterial 16S rRNA genes. Legend: M: marker (100 base pair ladder, Fermentas, Vilnius, Lithuania)

cies are numbered arbitrarily and are presented in Figures 1, 2 and 3 respectively. Figures 4,5,6 represent the restriction patterns obtained when digital restriction is carried out with the same enzymes on published GenBank sequences. The combination of these patterns is designed as ARDRA profiles and these are listed in Table 1. For example, strains of the *M. tuberculosis* complex can be recognized by an ARDRA profile 1-1-1, while *M. goodii* strains have ARDRA profile 8-4-2. For some species the ARDRA pattern obtained with enzyme is already characteristic, e.g. *HhaI* 1 is observed only for species of the *M. tuberculosis* complex.

Most mycobacterial species could be readily identified by comparison of the obtained ARDRA profile with the profiles from Table 1. The species of the *M. tuberculosis* complex can not be differentiated on the basis of the 16S rDNA sequence, and therefore restriction digestion of this gene could not either. Most of the clinically relevant and the most abundant species were readily differentiated from each other. The following species could not be differentiated from each other after digestion with *HhaI*, *MboI*, *RsaI* and *BstUI*: *M. gastri* and *M. kansasii* (1'-4-1-1), *M. bohemicum*, *M. haemophilum* and *M. malmoense* (1'-4-1-3), MCRO6 and *M. nonchromogenicum* (2-6-4-7), *M. chelonae* group I, *M. abscessus* and *M. immunogenicum* (3-3-6-2'), *M. farcinogenes*, *M. fortuitum*, *M. senegalense* and *M. septicum* (4-1-6-7), *M. simiae* and *M. lentiflavum* (5-7-6-2'), *M. goodii* and *M. smegmatis* (10-1-2-2'), *M. tusciae* and *M. flavescens* (10-1-6-2'), and finally *M. genavense* and *M. triplex* (10-7-6-2').

During 1998–2000, approximately 3500 samples were sent to the laboratory for direct smear examination and mycobacterial culture. Of these, 151 specimens, from 149 patients, were culture positive, and 20% of these were also smear positive. Table 2 summarizes the obtained identifi-

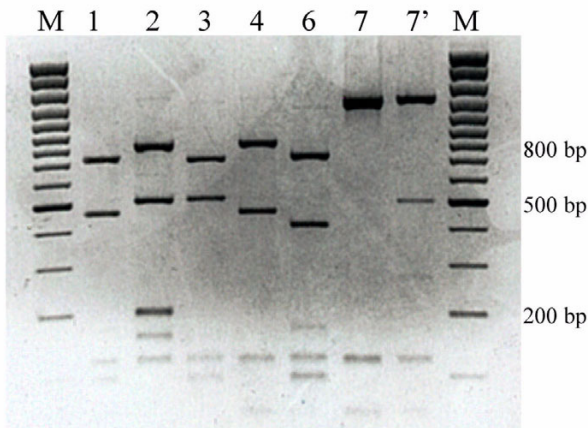


Figure 2
Mbol restriction patterns of amplified mycobacterial 16S rRNA genes. Legend: M: marker (100 base pair ladder, Fermentas, Vilnius, Lithuania)

cations. For 148 of the 151 isolates, identification by ARDRA was straightforward and was obtained after an average of 36 hours after receipt of the cultured strain. Only three difficulties were encountered. One isolate presented with the ARDRA profile 5-4-6, not present in the ARDRA library at that time. Sequencing lead to an identification as *M. interjectum*[8], a species that was not yet covered by the library. A second isolate was first misidentified as *M. xenopi* (profile 1'-4-3), but later on it was observed that the *HhaI* fingerprint differed clearly from the *M. xenopi HhaI* 1' pattern by its low molecular size fragments (Figure 1). This *HhaI* pattern was designated *HhaI* 1", resulting in the unique ARDRA profile 1"-4-3. The isolate was identified as *M. heckeshornense* by sequencing of the 16S rRNA gene, and can now also be identified by ARDRA. The third problematic isolate had ARDRA profile 1-1-3, again a profile that had never been observed for any mycobacterial strain studied thus far. Sequencing of the 16S rRNA gave a 99.8 % similarity to the 16S rRNA sequence of the type strain of *M. tuberculosis*. The sequence revealed a mutation at *E. coli* position 646 from A to G, abolishing the *RsaI* restriction site GTAC at that place. This mutation shifts the *RsaI* pattern 1 to *RsaI* pattern 3 because the two fragments of resp. 620 and 180 bp are replaced by a single fragment of 800 bp. Further morphological and biochemical tests revealed an identification as probably *M. africanum*, one of the species of the *M. tuberculosis* complex. It should be mentioned that *M. africanum* reference strains used in a previous study [12], were found to have the regular *M. tuberculosis* complex ARDRA profile 1-1-1.

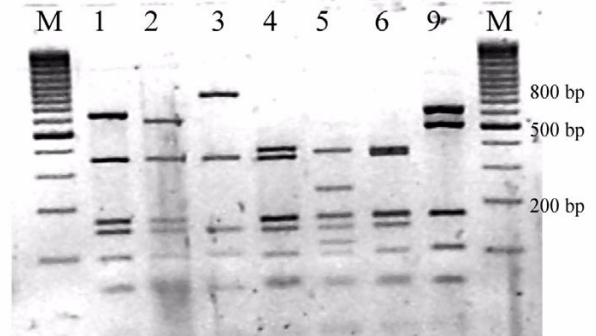
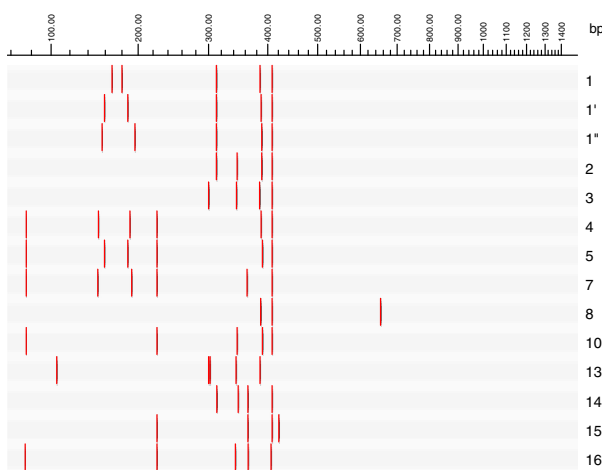


Figure 3
RsaI restriction patterns of amplified mycobacterial 16S rRNA genes. Legend: M: marker (100 base pair ladder, Fermentas, Vilnius, Lithuania)

To construct artificial ARDRA patterns for the recently described species, we applied the programme RFLP on the published Genbank sequences. The resulting, theoretically to be expected, ARDRA profiles are presented in Table 1.

M. tuberculosis was found to be the most prevalent species, with 40% of the isolates, followed by *M. gordonae* and *M. xenopi*, species mostly isolated from non-pulmonary samples with low clinical relevance.

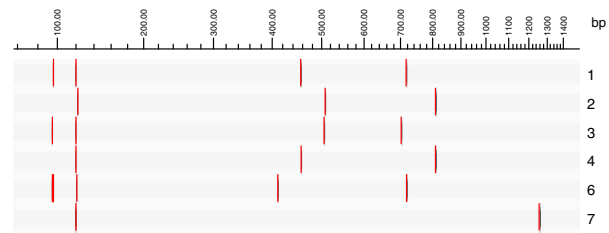
Similarity calculation of published sequences and of sequences obtained in this study (Figure 8) indicates that the *M. abscessus/M. chelonae* complex (*M. chehnae*, *M. abscessus* and *M. immunogenum*) clusters separately from all other mycobacteria. Portaels *et al.*[16] described the presence of four groups within the *M. abscessus/M. chelonae* complex, based on the 16S-23S spacer sequences, as used in the INNO-LiPA Mycobacteria kit (Innogenetics, Zwijnaarde, Belgium). Groups II and IV consisted of genuine *M. chelonae* strains, which were usually from environmental sources. These two groups have characteristic ARDRA profiles, namely 13-3-6 and 3-3-5. Group III, which was found to represent genuine *M. abscessus* and *M. chelonae* group I, which most probably corresponds with *M. immunogenum*, a *M. abscessus*-like species that was recently described as being frequently involved in bronchoscope related pseudo-outbreaks [17], can not be distinguished from each other by ARDRA (profile 3-3-6). Strains of groups I and III are usually isolated from clinical samples, with only group III strains (genuine *M. abscessus*) being pathogenic [16].



Pattern	CfoI restriction fragment lengths (bp)
1	408, 385, 312, 181, 168
1'	408, 388, 312, 187, 160
1''	408, 388, 312, 196, 157
2	408, 387, 347, 312
3	408, 385, 346, 300
4	408, 387, 225, 190, 155, 75
5	408, 387, 225, 187, 160, 75
7	408, 362, 225, 192, 152, 75
8	654, 408, 386
10	408, 389, 347, 225, 75
13	385, 346, 302, 300, 106
14	408, 360, 346, 312, 25
15	422, 408, 363, 225, 25
16	406, 364, 344, 225, 74, 25

Figure 4
HhaI (CfoI) restriction patterns of mycobacterial 16S rRNA genes, theoretically calculated using RFLP (Applied Maths) and published GenBank sequences. Graphical representation and table of restriction fragment lengths for each of the possible patterns.

M. tuberculosis was the most frequently cultured species (40% of all culture positives), followed by *M. goodii* (27%) and *M. xenopi* (15%) The latter two species were mostly isolated from non-pulmonary samples. 46% of the samples culture positive for *M. tuberculosis*, were auramine staining positive, and the average time of incubation until a positive liquid culture of *M. tuberculosis* was 12.9 days for the auramine positive samples and 20 days for auramine negative samples. The range of time to positivity for all *M. tuberculosis* positive samples was between 6 and 29 days. The average culture times for species other than *M. tuberculosis*, as far as data were available, are presented in Table 2.



Pattern	MboI restriction fragment lengths (bp)
1	710, 456, 121, 94, 51, 17
2	808, 508, 121, 17
3	702, 507, 120, 93, 17
4	808, 457, 121, 51, 17
6	718, 411, 121, 96, 94
7	1253, 120, 51, 17

Figure 5
MboI restriction patterns of mycobacterial 16S rRNA genes, theoretically calculated using RFLP (Applied Maths) and published GenBank sequences. Graphical representation and table of restriction fragment lengths for each of the possible patterns.

Discussion

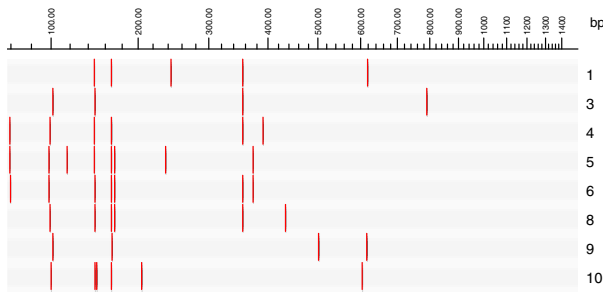
Restriction analysis of the amplified 16S rRNA gene, or amplified rDNA restriction analysis (ARDRA) was introduced into the Laboratory for Bacteriology of the Ghent University Hospital for the identification of cultured mycobacteria in 1993 [12]. Since then, several comparable approaches, based on restriction digestion of the amplified rRNA genes and spacer regions have been described [18–21]. During that period, this approach has been updated and refined. This was possible due to some technical changes, like increased quality control of gel electrophoresis and pattern interpretation and the use of primers specific for species of the order of the Actinomycetales instead of universal bacterial primers. Refinement was also possible because of the improvement of mycobacterial taxonomy and the possibility offered by PCR-RFLP techniques, like ARDRA, to easily adapt to this new information. Indeed, when new species are described, there is no need to develop new probes or primers. Instead, new ARDRA profiles can be easily added to the existing library. Also, ARDRA profiles for newly described species can be predicted by applying computer aided digestion of the available GenBank sequences, given the availability of sequences of sufficient quality [22].

ARDRA was found to be a useful tool for identification of mycobacterial isolates in a clinical routine laboratory, because of its speed – compared to phenotypic identification, its reliability, practical applicability, flexibility and the possibility to identify most nontuberculous mycobacteria together with and at the same cost as *M. tuberculosis*,

Table 1: Library of ARDRA profiles (combination of restriction patterns) obtained for mycobacterial species.

Species	Genbank Number	HhaI	MboI	RsaI	BstUI	Reference strains used ^a
<i>M. tuberculosis</i> complex	X52917	1	1	1	1	ITG 8021, IPB 92/0805
<i>M. conspicuum</i>	X88922	1'	1	2	2	
<i>M. intracellulare</i>	X52927	1'	2	2	3	ITG 5913, ITG 5917
<i>M. gastri</i>	X52919	1'	4	1	1	
<i>M. kansasii</i>	M29575	1'	4	1	1	ITG 8201
<i>M. bohemicum</i>	AJ277283	1'	4	1	3	
<i>M. haemophilum</i>	U06638	1'	4	1	3	ITG 3065
<i>M. malmoense</i>	AF152560	1'	4	1	3	ITG 940611
<i>M. szulgai</i>	X52926	1'	4	2	1	ITG 4981
<i>M. scrofulaceum</i>	X52924	1'	4	2	3	ITG 4988
<i>M. xenopi</i>	X52929	1'	4	3	2	ITG 4986
<i>M. heckeshornense</i>	AF174290	1'	4	3	4	
<i>M. marinum</i>	AF251565	2	1	1	1	ITG 1728
<i>M. asiaticum</i>	M29556	2	1	2	5	ITG 8182
<i>M. terrae</i>	X52925	2	1	4	2	ITG 4922
<i>M. ulcerans</i>	Z13990	2	1	9	1	ITG 724, ITG 1837
<i>M. avium</i>	AF306455	2	2	2	3	ITG 4991, ITG 2666
<i>M. botniense</i>	AJ012756	2	4	3	2	
<i>M. terrae</i> -like MCRO6 ^b	X93032	2	6	4	7	
<i>M. nonchromogenicum</i>	X52928	2	6	4	7	ITG 4980
<i>M. phlei</i>	M29566	3	1	6	2'	
<i>M. elephantis</i>	AJ010747	3	1	8	3'	
<i>M. chelonae</i> group IV ^c	AJ416940	3	3	5	2'	ITG 7701
<i>M. abscessus</i> group III ^c	AJ419970	3	3	6	2'	ITG 98-1296
<i>M. chelonae</i> group I ^c	AJ419969	3	3	6	2'	ITG 95-0026
<i>M. immunogenum</i>	AJ011771	3	3	6	2'	ITG 98-1289
<i>M. farcinogenes</i>	AF055333	4	1	6	7	
<i>M. fortuitum</i>	X52933	4	1	6	7	SLZ A046
<i>M. senegalense</i>	M29567	4	1	6	7	
<i>M. septicum</i>	AF111809	4	1	6	7	ITG 4166
<i>M. intermedium</i>	X67847	5	4	6	1'	
<i>M. interjectum</i>	AJ272088	5	4	6	2'	ITG 96-116
<i>M. heidelbergense</i>	X70960	5	4	8	2'	
<i>M. simiae</i>	X52931	5	7	6	2'	ITG 4485
<i>M. lentiflavum</i>	X80769	5	7	6	2'	
<i>M. peregrinum</i> ^d	X52921	7	1	6	9	ITG 99-2069, ATCC 14467
<i>M. gordonae</i>	M29563	8	4	2	5	ITG 7838
<i>M. wolinskyi</i>	Y12871	10	1	1	2'	
<i>M. species</i> ^a	AF028712	10	1	1	2'	
<i>M. goodii</i>	Y12872	10	1	2	2'	
<i>M. smegmatis</i>	AJ131761	10	1	2	2'	ITG 4995
<i>M. kubicae</i>	AF133902	10	1	6	1'	
<i>M. tusciae</i>	AF058299	10	1	6	2'	
<i>M. flavescens</i>	X52932	10	1	6	2'	VUB A016
<i>M. genavense</i>	X60070	10	7	6	2'	ITG 97-102
<i>M. triplex</i>	U57632	10	7	6	2'	
<i>M. chelonae</i> group II ^c	AJ419968	13	3	6	6	ITG 96-0295
<i>M. branderi</i>	X82234	14	1	1	9	
<i>M. celatum</i> ^e	L08169	14	1	1	10	
<i>M. triviale</i>	X88924	15	1	10	10'	
<i>M. aurum</i>	X55595	16	1	6	8	

a. ITG: Institute for Tropical Medicine, Antwerp, Belgium; IPB: Institute Pasteur du Brabant, Brussels, Belgium; SLZ: Streeklaboratorium Zeeland, Goes, the Netherlands; VUB: Free University of Brussels, Brussels, Belgium. b. Strains designated MCRO6 have been studied by Turenne *et al.*[22] and Torkko *et al.*[33]. c. Sequences determined in this study. Roman numbering according to Portaels *et al.*[16], who distinguished four groups within the *M. abscessus*/*M. chelonae* complex, based on the 16S–23S rRNA spacer region. d. The GenBank sequence (AF058712) of strain ATCC 14467, submitted to GenBank as *M. peregrinum*, did not cluster within the *M. fortuitum* complex (Figure 8) and was highly similar to sequence Y12871 (*M. wolinskyi*). Moreover, the ARDRA profile calculated from sequence AF058712 (10-1-1-2') did not correspond with the profile we obtained for strain ATCC 14467 (4-1-6-7). The sequence obtained in this study from strain ATCC 14467 (submitted as AJ422046) was identical to the *M. fortuitum* sequence X52933. Sequence AF058712 is indicated as *M. species* in the table. e. Received as *M. xenopi*.



Pattern	RsaI restriction fragment lengths (bp)
1	616, 355, 167, 148, 99, 53, 16
2	556, 355, 167, 148, 99, 60, 53, 16
3	788, 355, 148, 101, 53, 16
4	391, 355, 167, 167, 148, 99, 59, 55, 16
5	373, 237, 171, 167, 147, 117, 98, 59, 53, 16
6	373, 355, 171, 167, 148, 98, 60, 53, 16
8	433, 355, 171, 167, 148, 99, 53, 16
9	616, 502, 167, 102, 53, 16
10	604, 205, 167, 150, 148, 100, 53, 16

Figure 6
RsaI restriction patterns of mycobacterial 16S rRNA genes, theoretically calculated using RFLP (Applied Maths) and published GenBank sequences. Graphical representation and table of restriction fragment lengths for each of the possible patterns.

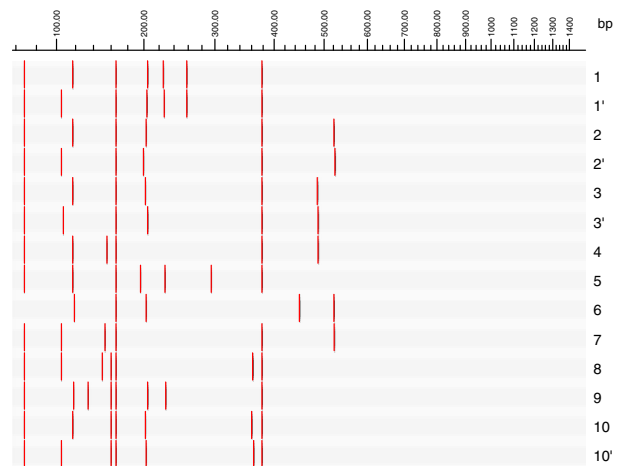
at an affordable price. Of the 151 isolates during the last three years, 148 could be identified without problems. The other three isolates, respectively *M. interjectum*, *M. heckeshornense* and an *M. africanum*-like strain, should be identifiable when met again in the future, since they presented with specific ARDRA profiles.

Practical applicability of ARDRA

The theoretical turnaround time of ARDRA is 6 hours, and the average identification time in practice during this study was 36 hours. It should be emphasized that the technique was not fully implemented in the routine laboratory, but was carried out by the research laboratory technicians, which means that the practical turnaround time should be far less than 36 hours in a routine diagnostic laboratory. Technically, ARDRA is nondemanding, comprising only basic molecular biology techniques like simple DNA extraction, PCR, restriction digestion and submarine agarose gel electrophoresis.

General considerations

We have addressed previously the several limitations of molecular biology based detection in diagnostic bacteriology [23]. Others agree that the expectations that DNA amplification technologies would supplant microscopy, accurately predict culture results and provide an immedi-



Pattern	BstUI restriction fragment lengths (bp)
1	378, 258, 227, 205, 166, 117, 68, 36
1'	378, 258, 227, 204, 166, 105, 68, 36
2	521, 378, 203, 166, 117, 68
2'	521, 378, 200, 166, 105, 68
3	485, 378, 202, 166, 117, 68, 35
3'	485, 378, 205, 166, 117, 68, 36
4	487, 378, 166, 155, 117, 68, 54, 36
5	378, 294, 228, 196, 166, 117, 68
6	521, 448, 203, 166, 119
7	522, 378, 166, 153, 105, 68, 48
8	378, 362, 166, 160, 150, 105, 68, 50
9	378, 229, 205, 166, 160, 134, 117, 68
10	378, 360, 202, 166, 160, 117, 68
10'	378, 363, 203, 166, 160, 105, 68

Figure 7
BstUI restriction patterns of mycobacterial 16S rRNA genes, theoretically calculated using RFLP (Applied Maths) and published GenBank sequences. Graphical representation and table of restriction fragment lengths for each of the possible patterns.

ate definitive diagnosis were premature and that these claims have to be replaced with a more realistic view of the limitations and of the practical value of molecular diagnostics of tuberculosis [10,24,25]. Also the expectation that susceptibility would be carried out solely by means of DNA technology, had to be moderated [26]. Despite the fact that during the last ten years a tremendous effort, both in academic and commercial research, has been put into the applicability of nucleotide amplification techniques for the detection of mycobacteria directly from clinical samples, the CDC approved application of these techniques only for smear positive samples. This implicates that for 52% of the culture positive samples with *M. tuberculosis* encountered in this study, DNA technology would not have accelerated detection, since microscopy

Table 2: Distribution of the *Mycobacterium* species during 1998–2000 in the Ghent University Hospital, number of smear positives and time to positivity of culture.

<i>Mycobacterium</i> species	Number of isolates (%)	Number of samples positive on direct smear ^a (% of culture positive)	Average time until positive culture in days (smear positives; negatives)
<i>M. tuberculosis</i> complex	61 (40)	26 (46 ^b)	16.7 (12.9; 20)
<i>M. avium</i>	12	2	6.5
<i>M. heckeshornense</i>	1	1	32.5
<i>M. chelonae</i>	4	0	18
<i>M. fortuitum</i>	2	0	35.5
<i>M. gordonae</i>	41 (27)	1	22.3
<i>M. interjectum</i>	1	0	30.5
<i>M. intracellulare</i>	1	0	11.6
<i>M. kansasii/M. gastri</i>	2	0	No data
<i>M. malmoense</i>	1	0	No data
<i>M. szulgai</i>	3	0	20.3
<i>M. xenopi</i>	22 (15)	0	38.1
Total	151	40 (26)	

^a: No data for 6 of the 61 samples. All six of these samples were *M. tuberculosis* culture positive. ^b: Calculated as 26 positives on a total of those 56 culture positives for which data on smear positivity were available.

was negative. Recently, AMTD2 (GenProbe) gained FDA approval for testing smear negative sputa, but the cost of the technique keeps limiting its use to only those smear negative samples with strong clinical suspicion of tuberculosis. Moreover, since this kind of direct detection amplification technology is technically demanding or requires specialized equipment and kits, many laboratories carry out these tests only at well-set time intervals [e.g. [11]], delaying diagnosis with several days on average, and as such losing some of the time gain offered by these direct detection methods. As a final remark, one should keep in mind that direct detection without direct antimicrobial susceptibility testing does not obviate the need for culture after all.

ARDRA compared to other culture based genotypic identification techniques

ARDRA and other gene restriction techniques [14,15,21,27,28] have been developed as a practical shortcut to full sequence determination. From this study and others it is clear that the discriminatory power of these RFLP approaches for identification of mycobacteria is almost as high as that of sequencing. The discriminatory power and reliability of a commercially available rRNA-spacer based hybridisation assay (INNO-LiPA Mycobacteria) is high, but again this approach is somewhat more laborious and more expensive than in house PCR-RFLP-based techniques. Restriction digestion of a 439 bp stretch of the *hsp65* gene for identification of mycobacteria was described almost simultaneously with ARDRA and designated PCR-RFLP Analysis (PRA) [15]. Several laboratories have published their experience using this technique [e.g.

[14,27,28]]. Possible drawbacks of *hsp65* gene restriction analysis are the smaller sized restriction fragments and the higher intraspecific variability, which may make interpretation more difficult. The small size differences have led to the use of polyacrylamide gel electrophoresis [27], which is less practical than agarose gel electrophoresis and the interpretation difficulties in general have led to reconsideration of the *hsp65* gene restriction profiles used thus far [28]. Comparable remarks can be made for PCR-RFLP analysis of the rRNA spacer region [21].

Future developments

At present, in an effort to have the best of both worlds, we are performing a double PCR, directly on smear positive, decontaminated samples, extracting DNA with the commercially available QiaAmp Tissue kit (Qiagen, Hilden, Germany). One PCR attempts to amplify the full length 16S rRNA gene (1500 bp), which can be used for ARDRA, at an annealing at 55°C, and one PCR amplifies a 123 bp region of the IS6110 at an optimal annealing of 68.6°C [29]. Both PCRs are carried out simultaneously in a T-gradient thermocycler (Biometra, Göttingen, Germany), programmed to have both annealing temperatures in the 96 well block. In case of the presence of the 123 bp fragment on an agarose gel, the identification of *M. tuberculosis* is completed, and can optionally be confirmed with ARDRA. Due to the higher sensitivity of the IS6110 PCR compared to the rDNA PCR, we could amplify the IS6110 fragment of *M. tuberculosis* directly from all smear positive, *M. tuberculosis* culture positive samples thus far. In case only the 16S rDNA fragment of 1500 bp is present, the absence of *M. tuberculosis* can be confirmed by ARDRA which

also immediately provides with the identification of the *Mycobacterium* species other than tuberculosis. Thus far, this yielded in all cases an identification as a nontuberculous strain, confirming the absence of *M. tuberculosis*. Also in case both amplifications of the smear positive sample remain negative, this can be interpreted as the absence of *M. tuberculosis*. A positive culture is then awaited to identify the nontuberculous organism with ARDRA.

Taxonomical considerations

This study also confirmed the robustness of ARDRA based identifications. For example, for the first study [12], we received strains identified by reference laboratories as *M. avium* and *M. xenopi*, using phenotypic methods. During the first study already, some strains that had been sent as *M. avium* could be shown by ARDRA (and subsequent confirmation by another laboratory) to be *M. scrofulaceum*. For *M. xenopi*, ARDRA indicated the presence of several groups. In the meantime mycobacterial taxonomy has been refined and the groups we indicated as *M. xenopi* A and *M. xenopi* B [12] appear to correspond to genuine *M. xenopi*, resp. *M. celatum* (ITG 6147)[30], as becomes apparent when applying the programme RFLP on the 16S rRNA sequence data we obtained. Similarly, strains that we classified as *M. fortuitum* A and B [12], appear to correspond to *M. fortuitum* subsp. *fortuitum*, resp. *M. peregrinum*.

Materials and Methods

Strains

A collection of well characterized reference strains belonging to different mycobacterial species was used to create an ARDRA profile library. The strains used are listed in Table 1. The 151 clinical strains used in this evaluation were collected during the period between January 1998 and December 2000 in the routine clinical laboratory of the Ghent University Hospital.

Processing and culturing of the samples

Decontamination of the samples was done by mixing 1 ml of sample with 1 ml of decontamination buffer (3% NaOH/N-acetyl L-cysteine (NALC)). After 15 min of incubation at room temperature the mixture was neutralized by adding 40 ml of 0.067 M phosphate buffer (pH 6.8), followed by centrifugation at 11600 g during 15 min. The supernatant was removed and part of the pellet was used for auramine staining and microscopy. The remaining pellet was suspended in 1 ml of phosphate buffer and used for inoculation of culture media. 100 µl was used for inoculation of a solid medium (Ogawa, Sanofi-Pasteur, Marnes la Coquette, France) and 500 µl for the inoculation of a liquid medium. During this study the automated liquid culture system was changed from the Bactec system (Becton Dickinson, Cockeysville, Md.) to the 3D BacT/Alert system (Organon Teknika, Boxtel, The Netherlands).

DNA extraction

Starting from liquid culture, 500 µl of a positive culture was transferred to a 1.5 ml screw cap Eppendorf tube. After centrifugation at 13.000 rpm for 15 minutes, the supernatant was removed and the resulting pellet was resuspended in 50 µl TE buffer (100 mM Tris-HCl – 10 mM EDTA, pH 8.0). The mixture was heated for 30 minutes at 95°C, followed by a freezing step at -20°C for at least 30 minutes. Starting from solid culture, a loopful of a bacterial colony was suspended in 500 µl of TE buffer. The mixture was heated at 95°C for 30 minutes, followed by a freezing step at -20°C for at least 30 minutes. Prior to PCR, DNA extracts were thawed at 4°C and centrifuged shortly to pellet the debris.

ARDRA for mycobacteria consists of the amplification of the 16S rRNA gene, followed by separate restriction digestion with *HhaI*, *MboI* and *RsaI*. The combination of the three obtained fingerprints is designated an ARDRA profile which can be compared with a library of ARDRA profiles, obtained from well-identified mycobacterial strains. In some cases, more discriminatory identification is possible by additional restriction with *BstUI*.

Amplification of the 16S rRNA gene

The primers used to amplify the full length 16S rRNA gene (approximately 1500 bp) were MBUZ1 (GAC GAA CGC TGG CGG CGT GCT TAA C) and MBUZ2 (CGT CCC AAT CGC CGA TC). These primers are designated to amplify only the 16S rRNA gene for species of the order Actinomycetales. The PCR mixture consisted of 25 µl Qiagen Mastermix (Qiagen, Hilden, Germany), 0.2 µM of each primer, 5 µl of DNA extract, and was adjusted to 50 µl with distilled water. Thermal cycling consisted of an initial denaturation of 5 min at 94°C, followed by three cycles of 1 min at 94°C, 2 min at 55°C and 1 min at 72°C, followed by 30 cycles of 20 sec at 94°C, 1 min at 55°C and 1 min 72°C, with a final extension of 7 min at 72°C, and cooling at 10°C.

Amplification of 123 bp of the IS6110 region was carried out as described [29] after DNA extraction from decontaminated sputum samples using the QiaAmp Tissue kit (QiaGen, Hilden, Germany).

Restriction digestion

The restriction enzymes used were *HhaI* (isoschizomer of *CfoI*) (Amersham Pharmacia Biotech Benelux, Roosendaal, the Netherlands), *MboI* (Fermentas, Vilnius, Lithuania), *RsaI* (Amersham Pharmacia). When necessary for further discrimination, digestion with *BstUI* (New England Biolabs, Beverly, Ma.) was carried out. Each 16S rDNA amplicon was divided in three separate tubes in aliquots of 10 µl, to which 10 U of the respective restriction enzymes were added, with 2 µl of the corresponding en-

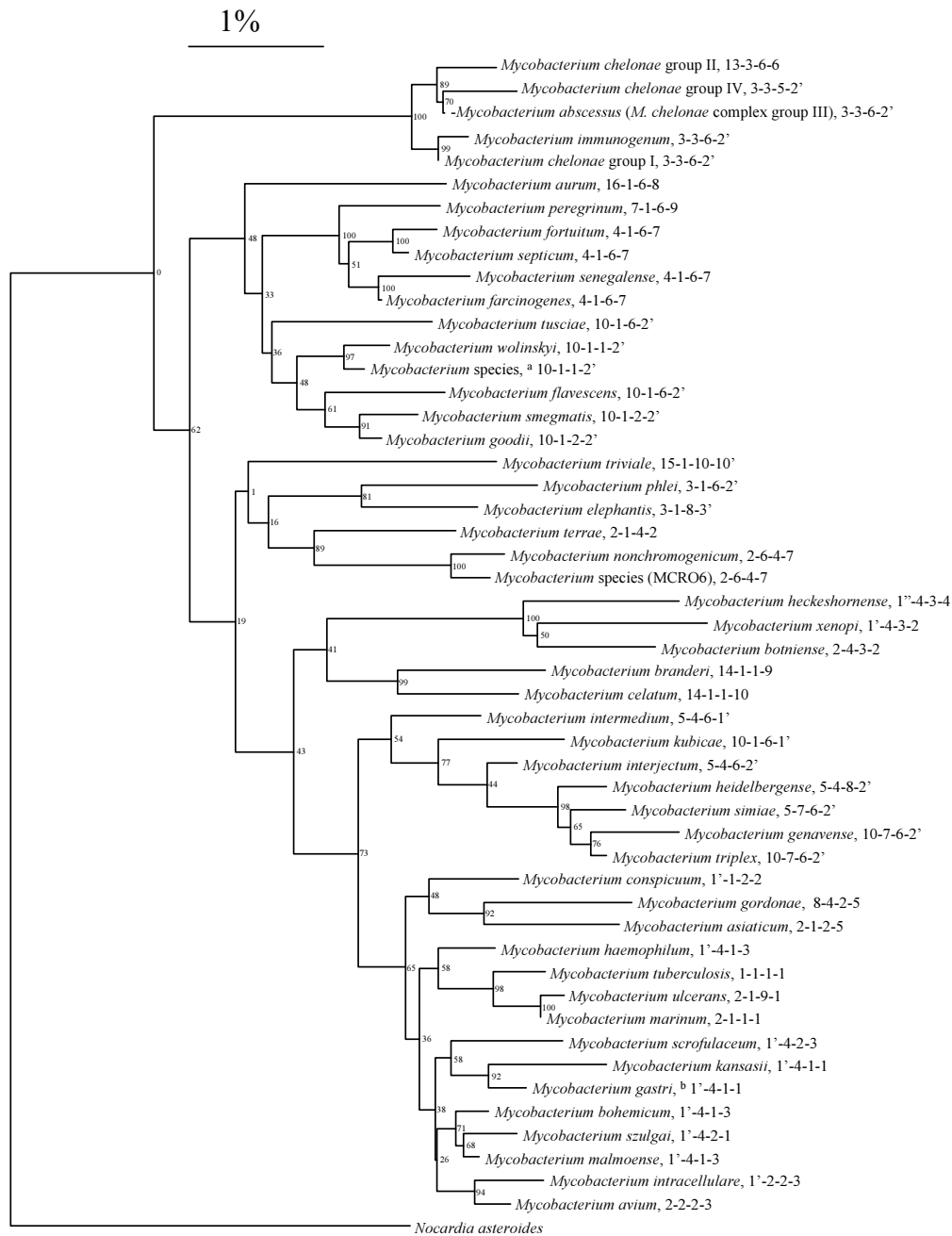


Figure 8

Neighbour-joining similarity tree for 16S rRNA gene sequences of most mycobacterial species. Legend: *N. asteroides* ATCC 49872 (Genbank Z82229) was used as the outgroup. Table 1 lists the GenBank accession numbers of the sequences used to construct this tree. ARDRA patterns for *HhaI*, *MboI*, *RsaI* and *BstUI* are listed after the species name. a: GenBank AF028712. Erroneously listed in GenBank as *M. peregrinum* (see also legend of Table 1). b: *M. gastri* clusters below 100% with *M. kansasii*, although it is generally agreed that the 16S rRNA gene sequences for *M. kansasii* and *M. gastri* are identical. This can be explained by the fact that the only available GenBank *M. gastri* sequence (X52919) contained several ambiguities. c: *M. lentiflavum*, initially not included in the manuscript is not presented in this tree. It clusters close to the branch including *M. heidelbergense*, *M. simiae*, *M. triplex* and *M. genavense*.

Table 3: Overview of the theoretical ARDRA profiles of the species that were newly described since the start of the evaluation period (1998), as obtained after computer aided digestion of the published sequences (see also Figures 4,5,6,7).

Species	Source of isolation	Reference	GenBank Accession Numbers	HhaI	MboI	RsaI	BstUI
<i>M. bohemicum</i>		[34]	AJ277284, U84502	1'	4	1	3
<i>M. botniense</i>	Water	[35]	AF012756	2	4	3	2
<i>M. elephantis</i>	Elephant	[36]	AF010747	3	1	8	3'
<i>M. goodii</i>	Human wounds	[37]	Y12872	10	1	(1)	2'
			Y12872 ^a	10	1	2	2'
<i>M. heckeshornense</i>	Lung disease	[38]	AF1174290	1	4	3	4
<i>M. kubicae</i>		[39]	AF133902	10	1	6	1'
<i>M. murale</i>	Walls of childrens daycare centre	[40]	Unpublished				
<i>M. septicum</i>	Catheter related bacteraemia	[41]	AF111809	4	1	6	7
<i>M. tuberculosis</i> subsp. <i>caprae</i>	Goats in Spain	[42]	AJ131120	1	1	1	1
<i>M. tusciae</i>		[43]	AF058299	10	1	6	2'
<i>M. wolinskyi</i>	Human wounds	[37]	Y12871, Y12873	10	1	1	2'

a. After resolving the ambiguity (N) present at *E. coli* position 95 in the Genbank sequence Y12872. An identical base (G) is found at this position in all mycobacteria.

zyme buffer (10× concentrated, final concentration 2×) and each restriction digestion mixture was adjusted to 20 µl with distilled water and incubated during 2 hours at 37°C in a heater.

Electrophoresis

The DNA restriction fragments were electrophoresed in a 2.5% agarose electrophoresis gel, containing 2% Methaphor (FMC Bioproducts, Rockland, Me.) and 0.5% MP agarose (Roche) in the presence of ethidium bromide (50 ng/ml). The gels were photographed and the fingerprints were compared visually with the overview gels (Figures 1, 2 and 3).

16S rDNA sequencing and comparative analysis

A fragment of the 16S rRNA gene (corresponding to positions 10-1507 in the *Escherichia coli* numbering system) was sequenced as described previously [31]. Sequencing primers were MB UZ1 (GACGAACGCTGGCGGCGTGCTTAAC, *E. coli* position 27-50), MB UZ2 (CGTCCCAATCGCCGATC, 1493 – 1476), MBP1 (CCG-GCCAACACTACGTGCCAGC, 502 – 522), MBP2 (CTGGAATTCCTGGTGTAGCGG, 673 – 693), MBP3R (GCATGTCAAACCCAGGTAAGG, 1006 – 986) and MBP4R (CCACCTTCCTCCGAGTTGACC, 1185 – 1165)

The 16S rDNA sequences obtained in this study are indicated in Table 1. All steps of the comparative sequence analysis were performed by using the GeneBase software package (Applied Maths, St. Martens Latem, Belgium), as described [32]. First, pairwise alignment using UPGMA was carried out with a gap penalty of 100 %, a unit gap cost of 20 % and an ambiguity cost of 50 % of the mismatch cost. Subsequently, global alignment – with *N. as-*

teroides ATCC 49872 (Genbank Z82229) used as the outgroup – was carried out on the region corresponding to positions 67 through 1444 of the 16S rRNA gene of *E. coli*, with costs as above. Finally, a similarity matrix of the aligned sequences was constructed by global alignment homology calculation and a gap penalty of 20 %. The neighbour-joining method was used to construct the dendrogram based on this similarity matrix. Bootstrap values were calculated.

Theoretical calculation of restriction patterns was done by means of RFLP (Applied Maths), which makes it possible to obtain restriction patterns using sequences in EMBL format, for every restriction enzyme. The programme Gel-Compar (Applied Maths) was then used to display the obtained fingerprints.

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Application and Evaluation of the Interlaboratory Reproducibility of tRNA Intergenic Length Polymorphism Analysis (tDNA-PCR) for Identification of *Streptococcus* Species

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The discriminatory power, speed, and interlaboratory reproducibility of tRNA intergenic length polymorphism analysis (tDNA-PCR) combined with capillary electrophoresis was evaluated for the identification of streptococci. This method was carried out in three different laboratories under highly standardized conditions for 54 strains belonging to 18 different species. It was concluded that interlaboratory reproducibility of tDNA fingerprints produced by means of capillary electrophoresis was sufficiently high to permit the exchange between different laboratories and the construction of common libraries which can be consulted for comparison with fingerprints obtained independently in separate laboratories. In a second step, 17 other species were included in the study and examined in one of the participating laboratories. All *Streptococcus* species studied, except *S. mitis*, *S. oralis*, *S. parasanguinis*, *S. pneumoniae*, *S. thermophilus*, and *S. vestibularis*, showed distinguishable tDNA fingerprints. A database of well-characterized strains was constructed to enable computer-aided identification of unknown streptococcal isolates.

Traditionally the clinically most important *Streptococcus* species have been identified by Lancefield carbohydrate antigen detection and the application of a few biochemical or physiological tests. Difficulties arise when less-prevalent species are to be dealt with. Lancefield groups are not species-specific (5, 10), and certain species groups (2) are notoriously difficult to differentiate phenotypically (8).

A number of genotypic methods have been evaluated for the identification of streptococci: amplified ribosomal DNA restriction analysis (7, 9), amplification of *ddl* genes (6), and sequencing of the MnSOD gene (13). tRNA intergenic length polymorphism analysis (tDNA-PCR) (15) has been used not only for the differentiation of streptococcal species (3, 12) but also for *Acinetobacter* (4, 16), staphylococci (11), *Listeria* (14), and enterococci (1). Thus far the interlaboratory reproducibility of this kind of genotypic identification technique has been ill studied although it is crucial with regard to the ability to compare fingerprints generated in different laboratories and with regard to the construction of publicly accessible DNA fingerprint data banks. Here we evaluated the interlaboratory reproducibility of tDNA-PCR in combination with capillary fluorescent electrophoresis and its suitability for identification in routine diagnostics.

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MATERIALS AND METHODS

Bacterial strains. Fifty-four BCCM-LMG culture collection strains (University of Ghent, K. L. Ledeganckstraat 35, B-9000 Ghent) belonging to 18 streptococcal species were used to standardize the method of tDNA-PCR and to evaluate its interlaboratory reproducibility (Table 1). The collection was extended with 47 strains of the BCCM-LMG culture collection belonging to 17 other streptococcal species (Table 2). Ten collection strains were subjected to blind testing in all three laboratories.

DNA preparation. Bacterial cells were grown overnight on Columbia agar (Gibco Life Technologies, Paisley, Scotland) with 5% ovine blood for 24 h at 37°C in a 5% CO₂-enriched environment and checked for purity. A 1- μ l loopful of cells was suspended in 20 μ l of lysis buffer (0.25% sodium dodecyl sulfate, 0.05 N NaOH) and heated at 95°C for 5 min. The cell lysate was spun down by brief centrifugation at 16,000 \times g and neutralized by adding 180 μ l of distilled water. The cell debris was removed by centrifugation at 16,000 \times g for 5 min. Supernatants were used as the DNA in the PCR or were frozen at -20°C until further use.

tDNA-intergenic PCR. PCR was carried out using outwardly directed tRNA gene consensus primers T5A (5' AGTCCGGTGCTCTAACCAACTGAG) and T3B (5' AGGTCGCGGGTTCGAATCC) as described by Welsh and McClelland (15). Reactions were carried out in a 10- μ l volume containing 9.1 μ l (dilution, 1.1) of High Fidelity Mix (Gibco Life Technologies). Primers were added to a final concentration of 0.1 μ M. Primer T3B consisted of a mixture of one-fifth fluorescent TET-labeled oligonucleotides and four-fifths nonlabeled oligonucleotides (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). A volume of 0.7 μ l of sample DNA was added (dilution, 1/15). After 2 min at 94°C, reaction mixtures were cycled 30 times in a Perkin-Elmer Cetus 9600 thermocycler with the following conditions: 30 s at 94°C, 1 min at 50°C, and 1 min at 72°C, without a final extension period. Reaction vials were then cooled to 10°C and kept on ice until used in electrophoresis.

Capillary electrophoresis. Twelve microliters of deionized formamide was mixed with 0.5 μ l of an internal size standard mixture containing 0.3 μ l of the GS-400 high-density size standard and 0.2 μ l of the GS-500 size standard, which both contain ROX-labeled fragments in the range of 50 to 500 bp. One microliter of tDNA-PCR product was added. The mixtures were denatured by heating at

TABLE 1. Strains used to standardize the tDNA-PCR method and to study the intra- and interlaboratory reproducibility

<i>Streptococcus</i> species	Strain no.
<i>S. agalactiae</i>	LMG14694 ^T , LMG14840, ^a LMG15081
<i>S. alactolyticus</i>	LMG14617, ^a LMG14872, LMG14808 ^T
<i>S. anginosus</i>	LMG14502 ^T , LMG14696, LMG17666
<i>S. bovis</i>	LMG15048, ^a LMG15055, LMG8518 ^T
<i>S. gallolyticus</i>	LMG14618, ^a LMG15049, LMG16802 ^T , LMG16880
<i>S. hyointestinalis</i>	LMG14579 ^T , ^a LMG14581, LMG14582
<i>S. hyovaginalis</i>	LMG14710 ^T , LMG14711, LMG14712
<i>S. mitis</i>	LMG14553, ^a LMG14557 ^T , LMG14552 ^T
<i>S. mutans</i>	LMG14558 ^T , ^a LMG14560
<i>S. oralis</i>	LMG14532 ^T , LMG14533, ^a LMG14534
<i>S. parasanguinis</i>	LMG14537 ^T , ^a LMG14538, LMG14539
<i>S. pyogenes</i>	LMG14237, LMG14238, LMG14700 ^T
<i>S. salivarius</i>	LMG13103, LMG13104, LMG11489 ^T
<i>S. sanguinis</i>	LMG14656, LMG14657, LMG14702 ^T
<i>S. suis</i>	LMG14181 ^T , LMG14182, LMG14643
<i>S. thoraltensis</i>	LMG13593, LMG14173, LMG14174
<i>S. uberis</i>	LMG14375, LMG14892, LMG09465
<i>S. pneumoniae</i>	LMG14545 ^T , LMG15155 ^a , LMG16738

^a Strains used in this study for blind identification of streptococci on the basis of their tDNA-PCR fingerprint pattern.

95°C for 3 min and placed directly on ice for at least 15 min (according to the recommendations of the manufacturer).

Capillary electrophoresis was carried out using an ABI-Prism 310 genetic analyzer (Applied Biosystems) at 60°C, at a constant voltage of 1.5 kV, and at a more or less constant current of approximately 10 mA. Capillaries with a length of 47 cm and diameter of 50 µm were filled with performance-optimized polymer 4. Electropherograms were normalized using Genescan Analysis software, version 2.1 (Applied Biosystems).

Data analysis. tDNA-PCR fingerprints were obtained as table files from the Genescan Analysis software and used in a software program developed at our laboratory (1). Using these sample files containing tDNA spacer fragment lengths (peak values) in base pairs, this program enabled us to construct manually a library which contains one entry for each species and whereby each entry consists of a number of numeric values representing the peak values in base pairs. The peak values in the library entries are the averages of the peak values obtained after testing different strains of each species, which are listed in Tables 1 and 2. The peaks retained for each entry are user selected, which means that the scientist takes the final decision about which peaks appear to be characteristic for each species. Negative values can be added to indicate that a certain peak must not be present in the fingerprint in order to be identified as a certain species. The similarity between the unknown fingerprint and a library entry is calculated with a coefficient, further referred to as dbp (differential base pairs): the number of fragments in common between the unknown fingerprint and the

species entry, divided by the total number of fragments of the species entry in the library. A peak position tolerance of 0.7 bp was used.

A distance matrix was calculated with the in-house software. Clustering analysis was done with the Neighbor module of the Phylip software (<http://evolution.genetics.washington.edu/phylip.html>), using the unweighted pair group method using arithmetic averages (UPGMA) algorithm. Ten well-characterized strains were tested blindly in all three laboratories and identified on the basis of their tDNA-PCR fingerprint by using this software.

Reproducibility testing. One *S. agalactiae* strain (LMG 14694^T) was tested 135 times by tDNA-PCR in order to evaluate the variation caused by differences in PCR mixture preparation, PCR cycling, and electrophoresis runs at and between three different laboratories.

One 10-fold PCR mixture was made in each of the three laboratories and the DNA template was added. This mixture was divided into 10 equal volumes of 10 µl (samples 1 to 10). Samples 1 to 5 were cycled immediately, and samples 6 to 10 were kept at -70°C. On each of the following 5 days, a 10-µl PCR mixture was freshly prepared (samples 11 to 15) and this tube was cycled together with one of the samples 6 to 10. In total, this resulted for each laboratory in 15 tubes with tDNA-PCR product obtained from the same strain. The content of all 45 products was then divided over three tubes, exchanged between labs, and run on the ABI Prism 310 genetic analyzer at each laboratory. This resulted in 135 tDNA-PCR fingerprints of the same strain. For another *S. agalactiae* strain, LMG 14840, tDNA-PCR was performed on three other thermocyclers: the iCycler

TABLE 2. Strains used to expand our collection and the tDNA fingerprint database

<i>Streptococcus</i> species	Strain no.
<i>S. acidominimus</i>	LMG 17755 ^T
<i>S. canis</i>	LMG 14833, LMG 14834, LMG 15890 ^T
<i>S. constellatus</i>	LMG 14503, LMG 14504, LMG 14507 ^T
<i>S. cricetus</i>	LMG 14508 ^T , LMG 14511
<i>S. crispatus</i>	LMG 14512 LMG 16320 ^T
<i>S. downei</i>	LMG 14514 ^T
<i>S. dysgalactiae</i>	LMG 14602, LMG 14602, LMG 15832, LMG 15738, LMG 15747, LMG 16026 ^T
<i>S. equi</i> subsp. <i>zooepidemicus</i>	LMG 15760, LMG 15888, LMG 16030 ^T
<i>S. gordonii</i>	LMG 14515, LMG 14517, LMG 14518 ^T
<i>S. iniae</i>	LMG 14520 ^T , LMG 14521, LMG 15978
<i>S. intermedius</i>	LMG 14548, LMG 14549, LMG 17840 ^T
<i>S. phocae</i>	LMG 16735 ^T , LMG 16736, LMG 16737
<i>S. porcinus</i>	LMG 14615, LMG 14837, LMG 15980 ^T
<i>S. rattus</i>	LMG 14559, LMG 14650 ^T , LMG 14651
<i>S. pluranimalium</i>	LMG 13649, LMG 14177, LMG 14385, LMG 14827
<i>S. thermophilus</i>	LMG 6896 ^T , LMG 7952, LMG 13564
<i>S. vestibularis</i>	LMG 13516 ^T , LMG 14645, LMG 14646

TABLE 3. Average values and standard deviations of six peak positions for all 122 fingerprints of *S. agalactiae* strain LMG 14694^T

Variable	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
Mean (bp)	54.65	62.40	88.90	148.02	241.24	252.70
SD (bp)	0.27	0.20	0.15	0.21	0.37	0.33
% SD	0.49	0.31	0.17	0.14	0.16	0.13
Minimum (bp)	54.22	62.04	88.56	147.5	240.58	251.58
Maximum (bp)	55.29	62.73	89.19	148.56	242.04	253.38

(Bio-Rad, Nazareth, Belgium), the PTC200 (MJ Research, Waltham, Mass.), and the Mastercycler (Eppendorf, Hamburg, Germany).

Reproducibility was evaluated by (i) calculation of the standard deviation of the peak values of the six predominantly present tDNA spacer fragment peaks and (ii) calculation of the similarity between all fingerprints by using the dbp coefficient and by clustering with the UPGMA algorithm, using the Phylip software.

RESULTS

Standardization. In the three laboratories, different protocols were tested in order to assess the best fingerprint results. This revealed that the PCR conditions which produced the most reproducible and discriminatory tDNA fingerprints in each laboratory were obtained with the PCR mixture composition and the PCR cycling conditions as described in Materials and Methods.

Reproducibility. Extensive testing of the reproducibility of tDNA-PCR was done by repeated amplification of one *S. agalactiae* strain (LMG 14694^T) in different laboratories using different reaction mixtures, thermal cycling runs, and capillary electrophoresis runs. One of the 45 PCR mixtures and 10 of the 135 electrophoresis runs failed to produce a fingerprint. This resulted in 122 tDNA fingerprints available for the reproducibility studies.

In its tDNA-PCR fingerprint, the *S. agalactiae* strain showed six predominant peaks, of which the mean peak values, standard deviations, and percent standard deviations (SD/peak value \times 100) for all 122 fingerprints are shown in Table 3. Only three samples (i.e., 2.5% of all cases), for all of which PCR was performed in one of the labs, lacked a single peak (of 241 bp).

Each of the 44 obtained PCR products was electrophoresed at the three different laboratories. For each PCR product, the ranges between maximal and minimal peak values obtained for the same DNA fragment were calculated. The lowest and highest ranges obtained for each of the six peaks are presented in Table 4. For the largest peak of 252.70 bp, a maximal range of 1.56 bp was observed.

For all samples for which capillary electrophoresis was carried out in the same laboratory, the mean peak values, standard deviations, and percent standard deviations were calculated for the six peaks and are summarized in Table 5. This

table also shows minimal and maximal peak positions obtained. In laboratory A (41 samples), the maximal standard deviation was 0.25 bp, for a mean peak value of 241.65 bp; in laboratory B (39 samples), it was 0.22 bp for a mean peak value of 252.56 bp; and in laboratory C (42 samples), it was 0.21 bp for a mean peak value of 240.97 bp.

In addition to these standard deviation calculations, the similarity values of the 122 capillary electrophoresis runs of the same strain carried out in three laboratories with varying factors were also calculated, using the dbp coefficient and clustering with UPGMA. The lowest similarity obtained with all 122 fingerprints was 87.8%.

It was observed that the addition of a final extension period of half an hour at 72°C increased the reproducibility of the fingerprints. Without final extension, double peaks differing in length by one bp frequently occurred. With a final extension period, the double peaks mostly disappeared to be replaced by the larger of the two peaks only.

For 12 samples cycled on the iCycler, 6 samples on the Mastercycler, and 6 on the PTC200, the standard deviations were 0.18, 0.11, 0.22, 0.14, 0.20, and 0.16 bp for the six peaks.

All 35 streptococcal species tested gave a tDNA-PCR pattern which consisted of three to seven large and reproducibly present (i.e., present in 97.5 to 100% of all cases) peaks and several small (i.e., less than 20% of the average peak height in a fingerprint) nonreproducibly present peaks, which were considered as noise. All 54 strains used in the reproducibility study gave reproducible fingerprints regardless of the laboratory in which the assay was performed.

Discriminatory power. Most species were easily distinguishable using tDNA-PCR (Table 6). The closely related species *S. bovis*, *S. alactolyticus*, and *S. gallolyticus* showed resembling but distinctive tDNA fingerprints. *S. mutans* and *S. gordonii* differed in one base pair of only one peak. The species *S. anginosus*, *S. constellatus*, and *S. intermedius* could be differentiated by the longer tRNA spacer fragments. *S. canis* and *S. dysgalactiae* fingerprints differed slightly in two peak values (Table 6). *S. oralis*, *S. mitis*, *S. parasanguinis*, and *S. pneumoniae* were not distinguishable on the basis of their tDNA pattern, nor were *S. vestibularis* and *S. thermophilus*. Some species were

TABLE 4. Mean values and minimal and maximal range of the peak values for a total of 35 triplets and 9 doublets obtained from three different laboratories

Variable	Mean values and range of peak values (bp)					
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
Peak avg	54.65	62.40	88.90	148.02	241.24	252.70
Minimal range	0.07	0.02	0.02	0.05	0.15	0.28
Maximal range	1.06	0.95	0.99	0.68	1.11	1.56

TABLE 5. Mean, standard deviation, and percent standard deviation of the six peaks in all fingerprints obtained in each laboratory

CE results in ^a :	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
Lab A (n = 41)						
Mean (bp)	54.39	62.48	88.72	148.22	241.65	253.05
SD (bp)	0.09	0.20	0.08	0.16	0.25	0.22
SD (%)	0.16	0.31	0.09	0.11	0.10	0.09
Minimal (bp)	54.22	62.04	88.56	148.00	240.82	252.08
Maximal (bp)	54.62	62.73	88.85	148.56	242.04	253.34
Lab B (n = 39)						
Mean (bp)	54.60	62.47	88.94	147.96	241.06	252.56
SD (bp)	0.13	0.17	0.07	0.14	0.16	0.22
% SD	0.23	0.27	0.08	0.09	0.07	0.09
Minimal (bp)	54.41	62.11	88.82	147.5	240.83	251.58
Maximal (bp)	54.88	62.68	89.06	148.29	241.74	253.38
Lab C (n = 42)						
Mean (bp)	54.95	62.26	89.04	147.86	240.97	252.46
SD (bp)	0.18	0.14	0.14	0.12	0.21	0.18
% SD	0.33	0.22	0.16	0.08	0.09	0.07
Minimal (bp)	54.20	61.53	88.80	147.64	240.58	252.06
Maximal (bp)	55.29	62.46	89.61	148.19	241.44	252.85

^a CE, capillary electrophoresis. n, number of samples.

divided into two groups on the basis of different tDNA patterns. This was the case for *S. iniae* and *S. porcinus*.

Use of the in-house software and a manually constructed library, which contained only the reproducible peak values, enabled straightforward differentiation between all of the species tested except between strains belonging to the species *S. pneumoniae*, *S. mitis*, *S. oralis*, and *S. parasanguinis* and between *S. thermophilus* and *S. vestibularis* strains. In three laboratories, identification of 10 well-characterized strains was attempted using tDNA-PCR and our software, without former knowledge of the species to which these strains belonged. All 10 strains were identified correctly in all laboratories.

A dendrogram obtained with the tDNA-PCR patterns is shown in Fig. 1. All strains belonging to the same species clustered together. The strains belonging to *S. mitis*, *S. oralis*, *S. parasanguinis*, and *S. pneumoniae* were found in the same cluster, as was the case for strains belonging to *S. thermophilus* and *S. vestibularis*. Strains belonging to the species *S. canis* and *S. dysgalactiae*, and to *S. anginosus*, *S. constellatus*, and *S. intermedius*, which show resembling but still different tDNA patterns, clustered together.

DISCUSSION

tDNA-PCR and capillary electrophoresis of the amplified DNA fragments already have been evaluated for the differentiation of *Listeria* species (14) and enterococci (1). To enable identification of a large number of strains, a software program which was described previously (1) has been developed at our laboratory. In the present study, the interlaboratory reproducibility of tDNA-PCR was evaluated in order to develop a fully exchangeable digital fingerprint database which can be consecutively extended with new fingerprints of species belonging to a wide array of genera.

For *S. agalactiae* strains, tDNA-PCR resulted in a fingerprint with six reproducibly present peaks. The standard deviation of the amplified tDNA spacer fragment lengths (peak values) was calculated for each of the six peaks obtained in 122 fingerprints

of strain LMG14694^T. The standard deviation of all samples ranged from 0.19 to 0.38 bp for peaks between 54 and 253 bp, which indicates that reproducibility with regard to peak values was extremely high.

The presence or absence of peaks must be caused by different PCR mixtures and/or PCR cycling runs and not by electrophoretic migration differences. The six DNA fragments which are most strongly amplified are reproducibly present in more than 97.5% of all cases. Therefore it can be concluded that peak presence reproducibility, i.e., PCR reproducibility, is high. Further, it becomes clear that the variation of peak values around the mean value is caused by electrophoretic run-to-run differences in and between laboratories (Table 4). The results show that electrophoresis of the same PCR product on another genetic analyzer can give peak position differences of more than 1 bp (the highest range found was 1.56 bp), which means that the difference is due to variation in migration and not to the addition or loss of a nucleotide during PCR. Therefore we conclude that the final variability between the obtained fingerprints was not caused by PCR mix preparation or by PCR cycling runs either in one laboratory or in different laboratories, but solely by differences in migration during capillary electrophoresis, whereby the largest difference occurred between ABI Prism 310 genetic analyzers in different laboratories. Nevertheless, the reproducibility was found to be very high and a peak position tolerance of less than 0.8 bp can be used to score corresponding fragments as identical.

In one laboratory, PCR mixtures were cycled on three other PCR cyclers and electrophoresis was carried out on the same genetic analyzer. The standard deviations for the six peaks ranged from 0.11 to 0.22 bp. This means that this PCR assay can be performed on the four PCR cyclers tested without the need for adjustment of the cycling conditions.

The observation that prolonged extension resulted in the disappearance of double peaks can be explained by the fact that this enables the *Taq* polymerase to add an extra A to most

TABLE 6. tDNA-PCR library constructed manually and containing numeric values that represent lengths of amplified tDNA spacers that ought to be present or absent in the fingerprint of an unknown strain in order to be identified as a certain species

<i>Streptococcus</i> species entry	Length of fragments considered (bp) ^a
<i>S. acidominimus</i>	65.1, 75, 90.3, 103.9, 165.5, 261.5
<i>S. agalactiae</i>	54.8, 62.5, 89, 148, 241, 252.5
<i>S. alactolyticus</i>	55.8, 63.5, 154, -155, 253.8
<i>S. anginosus</i>	77, 88, 155.3
<i>S. bovis</i>	55.8, 63.5, 154, -155, -241, 255
<i>S. canis</i>	92, 164, 264.5
<i>S. constellatus</i>	88, 155.3
<i>S. cricetus</i>	72.6, 81, 165.5, 260.5
<i>S. crispatus</i>	56.3, 79.7, 90, 152, 251
<i>S. downei</i>	71.8, 166.5, 261.3
<i>S. dysgalactiae</i>	92, 163.4, 263.5
<i>S. equi</i> subsp. <i>zooepidemicus</i>	56.4, 63.5, 84.7, 92.8, 171.8, 234.6, 272.3
<i>S. gallolyticus</i>	55.8, 63.5, 155, -241, 255
<i>S. gordonii</i>	77, 88, 159.7
<i>S. hyointestinalis</i>	61.2, 69, -88.6, 159.6, 255.1
<i>S. hyovaginalis</i>	-69, 88.6, 147.8, 160.2, 255.6
<i>S. iniae 1</i>	130.5, 150, 167.5, 265.3
<i>S. iniae 2</i>	130.5, 150, 169.5, 267.4
<i>S. intermedius</i>	77, 88, 155.3, 253.7
<i>S. mitis/oralis/parasanguinis/pneumoniae</i>	63.5, 84.5, 150
<i>S. mutans</i>	77, 88, 158.2
<i>S. parauberis</i>	66, 115.7, 165.5, 260.7, 290.5
<i>S. phocae</i>	56.3, 98.4, 165.3
<i>S. pluranimalium</i>	74.1, 99.5, 141, 162, 263
<i>S. porcinus 1</i>	148.3, 171.5, 242, 266.5, 318.1
<i>S. porcinus 2</i>	151.6, 168, 245, 263.3, 322.5
<i>S. pyogenes</i>	80.6, 92, 166.5, 266.5
<i>S. rattus</i>	56.3, 67.5, 261.6, 266.7
<i>S. sanguinis</i>	65.3, 92.7, 161.5, 258.6
<i>S. salivarius</i>	78.3, 148.3
<i>S. suis</i>	57.5, 65.5, 75.3, 92.8, 106.6, 162.2, 259.6
<i>S. thermophilus/vestibularis</i>	70.5, 79.5, 148.2, 247.5
<i>S. thoralensis</i>	74, 83.2, 166.5, 258, 271.9
<i>S. uberis</i>	117.5, 133.5, 168.5, 263

^a Negative values indicate the length of tDNA spacers that should be absent in the fingerprint.

of the PCR fragments, causing these to outnumber the peaks without an additional A.

To be able to compare a large number of patterns, a software program was developed in our laboratory. It takes into account only peak values, not peak intensities. Importantly, extra peaks which are caused by electrophoretic impurities or other unknown factors are ignored by the approach used here and therefore cannot influence the identification results. Variability in peak positions due to electrophoretic differences can be compensated by enlarging the position tolerance in the software. Still, visual checking of the patterns to confirm the results is advised.

tDNA-PCR seems to be suited for the identification of most streptococcal species. However, *S. mitis*, *S. oralis*, *S. parasanguinis*, and *S. pneumoniae*, belonging to the clinically important viridans streptococci and to one phylogenetically closely related *S. mitis* species group (2), showed highly resembling patterns (see Table 6) and were not distinguishable. Recently, Degheldre et al. (3) have evaluated the discriminatory power of tDNA-PCR for the differentiation of viridans streptococci with separation of fragments on an ALFexpress DNA sequencer. Apparently, the patterns they found are not similar to those obtained in our study, because of the presence of more large fragments and fewer small ones. In their study, the large

fragments enabled discrimination within the *S. mitis* group. The cause of this disagreement is not clear.

tDNA-PCR is very rapid and relatively easy to perform. In a PE 9600 thermocycler, 96 samples can be run at once. Starting from a single colony, DNA extraction takes about 3 h for 96 strains. The preparation of the tDNA-PCR mixtures in separate tubes and addition of the sample DNA takes about 1 h and the PCR run itself takes 2 h. During this run, the genetic analyzer ABI Prism 310 capillary electrophoresis apparatus can be prepared. Denaturation and preparation of the PCR products takes about 30 min. The electrophoresis run takes about half an hour per sample, but with the use of different dyes for labeling primers, three samples can be run at the same time. Quality control of the obtained profiles by means of GeneScan analysis and comparison of the tDNA-PCR profiles of the unknown strains with the database takes another hour. Summarized results for all 96 strains can be available within 25 h if the three-dye technology is used. Obviously, taking fewer samples at once will reduce the manipulation time, which makes it possible to have the first electrophoresis results within 8 h after colony picking.

The cost, including culture, DNA extraction, PCR, and capillary electrophoresis, was calculated as \$2.50 per strain. Given the possibility for automation, the broad applicability of

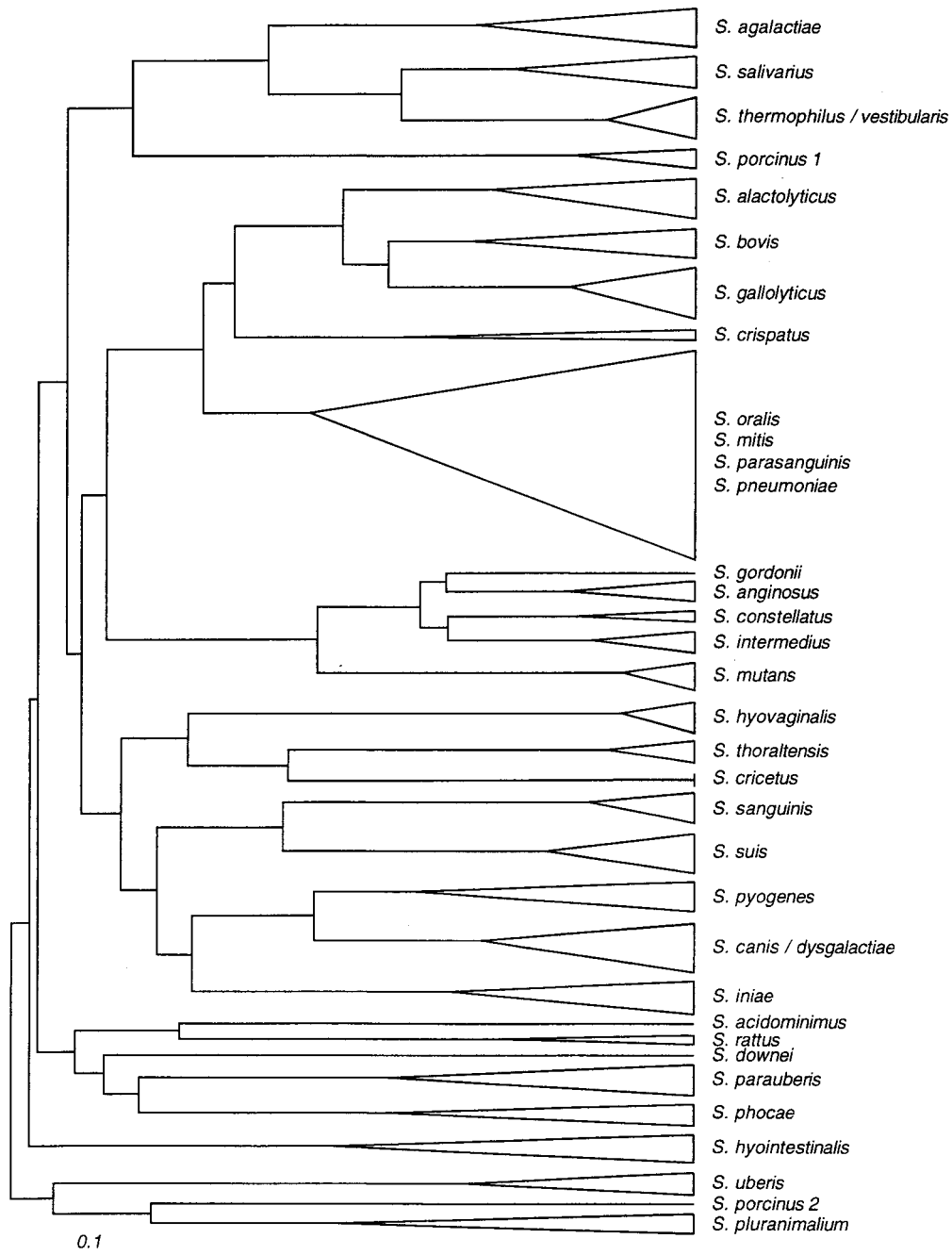


FIG. 1. Dendrogram obtained after similarity calculations between the tDNA fingerprints of streptococci. The bar represents a distance of 10%.

tDNA-PCR for species identification, and the interlaboratory exchange of data due to its high reproducibility, tDNA-PCR could be developed as a routinely applicable genotypic identification technique.

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Methodology article

Identification of cultured isolates of clinically important yeast species using fluorescent fragment length analysis of the amplified internally transcribed rRNA spacer 2 region

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Abstract

Background: The number of patients with yeast infection has increased during the last years. Also the variety of species of clinical importance has increased. Correct species identification is often important for efficient therapy, but is currently mostly based on phenotypic features and is sometimes time-consuming and depends largely on the expertise of technicians. Therefore, we evaluated the feasibility of PCR-based amplification of the internally transcribed spacer region 2 (ITS2), followed by fragment size analysis on the ABI Prism 310 for the identification of clinically important yeasts.

Results: A rapid DNA-extraction method, based on simple boiling-freezing was introduced. Of the 26 species tested, 22 could be identified unambiguously by scoring the length of the ITS2-region. No distinction could be made between the species *Trichosporon asteroides* and *T. inkin* or between *T. mucoides* and *T. ovoides*. The two varieties of *Cryptococcus neoformans* (var. *neoformans* and var. *gattii*) could be differentiated from each other due to a one bp length difference of the ITS2 fragment. The three *Cryptococcus laurentii* isolates were split into two groups according to their ITS2-fragment lengths, in correspondence with the phylogenetic groups described previously. Since the obtained fragment lengths compare well to those described previously and could be exchanged between two laboratories, an internationally usable library of ITS2 fragment lengths can be constructed.

Conclusions: The existing ITS2 size based library enables identification of most of the clinically important yeast species within 6 hours starting from a single colony and can be easily updated when new species are described. Data can be exchanged between laboratories.

Background

Rapid and correct identification of the different clinically relevant yeast species has become more important because of several reasons. During the last decade, the impact and frequency of yeast infections has gained importance mainly due to an increased number of immunocompromised patients [1]. Furthermore, an increasing number of non-*C. albicans* yeast species are considered as potential agents of clinical infections [2]. Finally, the differences in susceptibility towards antifungal agents between the different species make that rapid yeast identification can be used as a first indication for efficient treatment.

Phenotypic identification relies on cell and colony morphology and on biochemical characteristics, but this approach is not always fully discriminative, may be time-consuming and requires specific expertise of the technicians [3]. Therefore several groups have explored the possibilities of PCR-based techniques for the differentiation between different yeast species. The use of species specific probes [4,5] or molecular beacons [6] resulted in very sensitive and very specific techniques but remains restricted to the species for which the probes are designed for. A more broadly applicable approach is based on PCR with universal fungal primers (directed towards conserved regions in the ribosomal region) and followed by either restriction analysis [7], sequencing [8] or size determination of the amplified fragment(s) [9,10].

Here we report an evaluation and extension of the technique published by Turenne *et al.* [9], whereby the rRNA Internally Transcribed Spacer Region 2 (ITS2), i.e. the region in between the fungal 5.8S and 28S rRNA genes, is amplified and its length is determined by fragment analysis on an ABI Prism 310 capillary electrophoresis system. Turenne *et al.* [9] showed that the length of the ITS2-spacer region was characteristic for most species and that it was identical for all strains within a species, although only a limited number of strains was used per species. By using one fluorescently labeled primer, the size of the amplicon can be determined by electrophoresis on an ABI Prism 310 capillary apparatus, which provides with the one base pair resolution that is needed to differentiate between ITS2-fragment lengths of closely related species. In this study, we also evaluated a simpler and more rapid DNA-extraction method and we applied ITS2-PCR on ABI Prism 310 on more strains and species than in the original publication.

Results

Using DNA-extraction based on boiling/freezing of the yeast colonies, the ITS2-PCR fragment could be amplified easily from all yeast strains. An initial ITS2 size based library was constructed using a panel of reference strains,

Table 1: Reference strains used in this study

Species	Reference strains*
<i>Blastoschizomyces capitatum</i>	IHEM 5091
<i>Candida albicans</i>	IHEM 3242, IHEM 3731, IHEM 3740, IHEM 6440
<i>C. dubliniensis</i>	IHEM 14280
<i>C. glabrata</i>	IHEM 4210, IHEM 4566, IHEM 5573, IHEM 9556
<i>C. guilliermondii</i>	IHEM 1067, IHEM 1879, IHEM 3283
<i>C. humicola</i>	AZB 98-043
<i>C. kefyr</i>	IHEM 4211, IHEM 4593, IHEM 5556
<i>C. krusei</i>	IHEM 4562, IHEM 5579, IHEM 6387
<i>C. lipolytica</i>	HHR A101315
<i>C. lusitanae</i>	IHEM 4593, IHEM 10293, IHEM 14663
<i>C. norvegensis</i>	HHR A211294
<i>C. parapsilosis</i>	IHEM 1001, IHEM 2052, IHEM 2305, IHEM 3270
<i>C. tropicalis</i>	IHEM 4222, IHEM 4666, IHEM 5609, IHEM 12085
<i>Cryptococcus albidus</i>	IHEM 3267, IHEM 4786, IHEM 6895
<i>C. laurentii</i>	IHEM 895, IHEM 5515
<i>C. neoformans</i> var. <i>gattii</i>	IHEM 4170
<i>C. neoformans</i> var. <i>neoformans</i>	IHEM 4165, IHEM 4171
<i>Geotrichum candidum</i>	IHEM 6284
<i>Malassezia furfur</i>	IHEM 3697
<i>Saccharomyces cerevisiae</i>	IHEM 3962, IHEM 14402, IHEM 14542, IHEM 17539
<i>Trichosporon asahii</i>	IHEM 9334, IHEM 17910
<i>T. asteroides</i>	IHEM 10214
<i>T. inkin</i>	IHEM 5824
<i>T. mucoides</i>	IHEM 13920
<i>T. ovoides</i>	IHEM 17913

*: IHEM: strains from the BCCM/IHEM Culture Collection, Mycology Section, Scientific Institute of Public Health, Brussels, Belgium; HHR: Heilig Hart Ziekenhuis, Roeselare, Belgium, AZB: St. Jan Akademisch Ziekenhuis, Bruges, Belgium.

belonging to most of the clinically important species (Table 1).

When amplifying the ITS2-PCR fragment, care was taken to label the same primer as used by Turenne *et al.* [9] and to use the same HEX-label, in order not to introduce artefactual length differences. Still, when the obtained fragment lengths were compared with those of Turenne *et al.* [9], differences were observed (Table 2). These could be explained by our use of dTTP, compared to the use of dUTP in the original publication [9]. Unpublished fragment lengths obtained at the laboratory of Turenne when using dTTP were in agreement with our findings (Turenne, pers. comm.).

Table 2: Overview of the different ITS2-fragment lengths observed and of the number of strains identified by means of ITS2-PCR.

Species	Number of strains tested (number used for calculation of the average length)	ITS2-fragment length (bp), calculated by capillary electrophoresis			Previously published lengths (bp)*[9]	Fragment lengths (bp) derived from Genbank sequences	Genbank accession number of sequences used
		Average	Range	Standard deviation			
<i>Blastoschizomyces capitatum</i>	1	250.7			248	251	AF455443
<i>Candida albicans</i>	116 (19)	281.6	281.0 – 282.6	0.37	279	284	AF217609
<i>C. dubliniensis</i>	1	286.3				289	AF218993
<i>C. glabrata</i>	94 (12)	362.6	362.4 – 362.8	0.15	360	365	AF218994
<i>C. guilliermondii</i>	8	323.2	322.7 – 324.2	0.46	321	325	AF218996
<i>C. humicola</i>	1	295.8					
<i>C. kefyri</i>	7	375.5	375.4 – 375.8	0.17	372	378	AF218997
<i>C. krusei</i>	10 (2)	285.3	285.2 – 285.4	0.13	282	294	L47113
<i>C. lipolytica</i>	3 (2)	180.6	179.9 – 181.1	0.62		190	AF218983
<i>C. lusitanae</i>	9 (4)	200.3	200.2 – 200.4	0.10	199	204	AF218970
<i>C. norvegensis</i>	3 (2)	267.1	266.6 – 267.1	0.62		318	AF333096
<i>C. parapsilosis</i>	36 (12)	254.1	253.5 – 255.4	0.60	251	259	U10989
<i>C. rugosa</i>	1	215.0	214.8 – 215.1	0.10		223	AF218971
<i>C. tropicalis</i>	34 (28)	270.6	269.3 – 271.2	0.61	269	272 273 274	AF219000 AF219001 AF218992
<i>Cryptococcus albidus</i>	3	353.3	352.4 – 354.7	1.26	350	353	AF218972
<i>C. laurentii</i> (environmental)	2	272.1	271.9 – 272.3	0.26		275	AF218974
<i>C. laurentii</i> (clinical)	1	307.9			306	310	AJ421006
<i>C. neoformans</i> var. <i>neoformans</i>	8 (4)	318.3	318.0 – 318.6	0.29	315	320	AF218975
<i>C. neoformans</i> var. <i>gattii</i>	1	320.0				321	AF196312
<i>Dekkera bruxellensis</i>	1	261.8				263	AF043502
<i>Geotrichum candidum</i>	3 (1)	193.4			192	195	AF157596
<i>Malassezia furfur</i>	1	494.4				500	AF246896
<i>Saccharomyces cerevisiae</i>	35 (25)	366.6	365.1 – 368.5	0.89	363	365 366 367	AF219005 AF219006 AF219007
<i>Trichosporon asahii</i>	2	301.0	300.8–301.4	0.50		304	AB018014
<i>T. asteroides</i>	1	298.6				304	AF444416
<i>T. inkin</i>	1	298.7				304	AF444420
<i>T. mucoides</i>	1	297.2				297	AF444423
<i>T. ovoides</i>	1	297.5				303	AF444439

*: Values obtained by using dUTP instead of dTTP for the PCR-based amplification reaction [9].

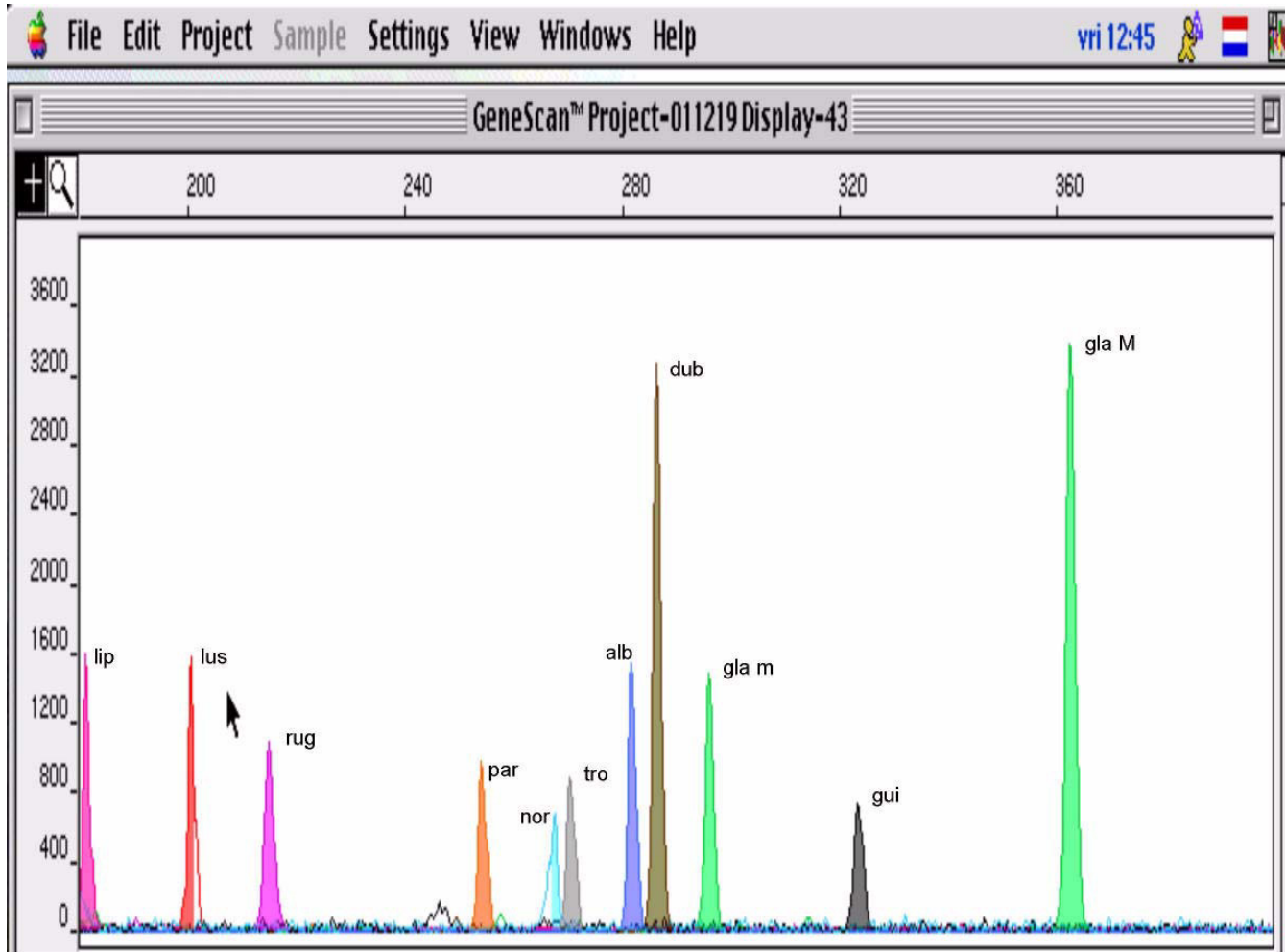


Figure 1
The ITS2-PCR fragment lengths observed for 10 *Candida* species (GeneScan Analysis screen, ABI Prism 310, Applied Biosystems).

The reproducibility of ITS2-PCR is apparent from Table 2. For example, for 19 *C. albicans* strains tested, the observed fragment sizes ranged between 281.0 bp and 282.6 bp (with an average value of 281.6 bp and a SD of 0.4 bp). Table 2 presents the ITS2-lengths observed for the different species and reference strains tested.

In one laboratory, we observed in a reproducible manner a smaller sized peak with lower intensity for half of the strains and for more than half of the species. An example is shown in Figure 1 for *C. glabrata*. However, using the same DNA-extracts, primer batch, commercially prepared PCR mixture and thermal cycling protocol, this additional fragment could not be observed in a second laboratory. When the primers of the second laboratory were used in the first laboratory, the additional fragments were ob-

served again. The only technical difference noticed between both laboratories were the thermal cyclers.

Of the 26 species, 22 could be identified unambiguously by scoring the length of the ITS2-region. In the genus *Trichosporon* however, no distinction could be made between the species *T. asteroides* and *T. inkin*, since the ITS2-fragment length is approximately 298.6 bp for both species, and between the species *T. mucooides* and *T. ovooides*, which both have an ITS2-fragment of approximately 297.5 bp.

Also the two varieties of *Cryptococcus neoformans* (var. *neoformans* and var. *gattii*) could be differentiated from each other due to a one bp length difference of the ITS2 fragment, although these findings should be further confirmed by inclusion of more strains.

The three *C. laurentii* isolates were split into two groups according to the ITS2-fragment length. The clinical isolate (IHEM, 1296, human mouth) had a fragment of 307.9 bp, while the two environmental isolates (from filter board of humidifier system (IHEM 0895) and from indoor air (IHEM 5515)), had an ITS2 length of 272.1 bp. The ATCC 18803 type strain, an isolate from palm wine, was found to yield a fragment of 306 base pairs, using dUTPs [9]. Sequencing of the ITS2 of the three strains identified them all as *C. laurentii*. Cluster analysis with Genbank sequences, published recently [11], revealed that the clinical isolate (IHEM 1296, Genbank AJ421004) clustered most closely to the type strain in Sugita's phylogenetic group I [11], in correspondence with the ITS2-PCR results, whereas the environmental strains (IHEM 0895: Genbank AJ421005 and IHEM 5515: Genbank AJ421006) clustered in phylogenetic group II, which contained mostly environmental strains and one isolate from the bronchi of a lung patient (Figure 1).

After construction of the library, a large collection of clinical isolates was identified both with ITS2-PCR and by phenotypic means, using Albicans ID plate (BioMérieux, Marcy-l'Etoile, France) and/or Auxacolor (Sanofi-Pasteur, Marnes-la-Coquette, France) and/or cellular and colony morphology. The number of additional strains tested for each species is listed in Table 2. Two isolates could initially not be identified by ITS2-PCR, because the observed ITS2-fragment lengths were not present in the library. According to biochemical and ITS2-sequencing data, the first isolate belonged to the species *Candida rugosa*, a species initially lacking in the library. The ITS2 fragment length value of 215.0 bp for *C. rugosa* was added to the database. Another isolate from a hematology patient and from syrup present in his room, had a previously not observed ITS-length of 261.8 bp and sequencing revealed an identity as *Dekkera (Brettanomyces) bruxellensis*, a species that had not been included in the library initially. The ITS2 fragment lengths obtained for 25 *S. cerevisiae* strains ranged between 365.1 and 368.5 bp (average 366.7 bp., SD 0.89 bp.), but no misidentifications occurred.

Clear differences could be observed between the length of the ITS2-region as derived from published sequences (theoretical length) and as calculated after capillary electrophoresis (estimated length) (Table 2). Although the estimated length is highly reproducible, it is consistently shorter than the theoretical length (except for *Blastoschizomyces capitatum*, *C. albidus*, *T. mucoides* and *S. cerevisiae*, where it is identical). A maximal difference of 9 bp was observed for *C. krusei*. For *S. cerevisiae*, the range of 3 bp observed for the estimated ITS2 lengths was also present in the theoretical lengths derived from the Genbank sequences.

Discussion

ITS2-PCR followed by size determination of the fragment length by capillary electrophoresis has been described as a possible tool for the identification of yeasts by Turenne *et al.* [9] and by Chen *et al.* [10]. The latter authors amplified a 50 bp longer stretch of the ITS2 region than did the former. Since ITS2-PCR fragments of different species may differ by only one bp, highly reproducible high resolution electrophoresis as provided by capillary electrophoresis on ABI Prism 310 is necessary.

In order to increase the speed of this approach we used a simple and rapid boiling/freezing based DNA extraction protocol, which proved to be highly efficient. Together, DNA-extraction, PCR and capillary electrophoresis took 3.5 hours. Thus, the total time to identification is 3.5 hours for the first sample, added with 45 minutes for each following sample, when using a single capillary apparatus like the ABI Prism 310. When a 16 capillary apparatus is used (ABI Prism 3100), the first 16 strains can be identified within 3.5 hrs. Besides speed and high discriminatory power, another advantage of this approach is that newly recognized species or species not yet present in the database can be added after appropriate initial identification, whereafter their identification by means of ITS2-PCR is straightforward. Given the identical values we obtained with unpublished ones by Turenne *et al.* [pers. comm.], it can be assumed that the data are exchangeable between laboratories, as also has been shown for other DNA-fingerprints obtained on ABI Prism 310 machines [12,13]. The lengths obtained for the amplified ITS2 fragments in two different laboratories during this study were indeed perfectly comparable. The only difference was an apparently artefactual additional peak of lower size and lower intensity in one laboratory, never observed in the other laboratory.

The variety of species that can be identified can be expanded and this can be achieved in a joint effort between laboratories. Moreover, laboratories not having constructed such a database their selves, can compare the data of their unknowns to a publicly available database, like the one published here. Care should be taken to use the same primer labeled in the same manner and to use the same size markers and size standard files (available upon request) to normalize the capillary electrophoresis runs, since minor differences between these can introduce migration and normalization differences.

The differences between the theoretical length (as obtained after counting the bases of the available ITS2 sequences) and the calculated length (as obtained after capillary electrophoresis) can be explained by the fact that electrophoretic migration is also partially sequence dependent, such that the calculated length will not always

match exactly the theoretical length. However, as long as the calculated length is reproducible from run to run and between laboratories, this observation poses no problem for the purpose of identification. Only for one species (*C. norvegensis*) the obtained length in this study was completely different from the one derived from the published GenBank sequence (Table 2).

Using ITS2-PCR, identification to species level is very efficient for the genera *Candida* and *Cryptococcus*, while four species of the five tested for the genus *Trichosporon* could be split into two groups only. However, the current taxonomy of *Trichosporon* needs further refinement, and our findings should be corroborated on additional, taxonomically well characterized strains.

The two different ITS2 lengths found for the *C. laurentii* strains in this study appear to correspond with a recently described phylogenetic subdivision within this species, that was based on the sequences of the 28S rRNA and of the ITS1 and ITS2 regions [11]. Thus, it appears that both groups can be differentiated simply by determination of the ITS2 length and that a distinction between environmental and clinical isolates might be possible. Although the ITS2-fragment lengths range between 365.1 and 368.5 basepairs for *S. cerevisiae* strains, identification remains possible since these fragment lengths are not encountered in other clinically important yeast species studied thus far.

When methods are used that are suitable for DNA-extraction directly from clinical specimens, it should be possible to detect and identify at once the different species that may be simultaneously present, since the fingerprint will consist of ITS2 fragments of different lengths, corresponding to the different ITS2-lengths for each species. However, when applied for direct detection, the minor peaks, as observed in one of the laboratories, could lead to problems in assessing the number and nature of the species present.

When ITS2-primers are labeled differently from e.g. tRNA-PCR primers [12–14], electrophoresis of bacterial tRNA-PCR and fungal ITS2-PCR can be carried out simultaneously.

Materials and Methods

Strains

A collection of 53 culture collection strains (Table 1) and 90 phenotypically well-identified strains, representing 24 species, was used to evaluate the technique and to set up an initial database. The technique and database were evaluated by identification of 242 clinical isolates from four Belgian hospitals (Ghent University Hospital, Heilig Hart Ziekenhuis Roeselare, Akademisch Ziekenhuis VUB Jette

and Akademisch Ziekenhuis St. Jan Bruges). In total, 385 strains belonging to 26 species (Table 2) were included.

DNA extraction

A boiling-freezing protocol was used for DNA-extraction. A 1 µl loopful of growth on Sabouraud agar (Becton Dickinson, Maryland, Ca.) was suspended in 500 µl distilled water and heated for 15 minutes at 95°C, immediately followed by freezing at -70°C for at least 15 minutes. Before adding the DNA-extract to the PCR mix, the samples were thawed at room temperature, and shortly centrifuged to pellet the cell debris still present in the extract.

ITS2-PCR

The ITS2-region was amplified with the following primers: ITS86 (GTG AAT CAT CGA ATC TTT GAA C-HEX) (fluorescently labeled) and ITS4 (TCC TCC GCT TAT TGA TAT GC) [9]. The ITS86 primer was used as a mixture of 10% fluorescently labeled and 90% non-fluorescently labeled primer in order to avoid out of range peak heights. The PCR reaction mix contained 12.5 µl Qiagen Mastermix (Qiagen, Hilden, Germany), 0.5 µM of each primer, 2.5 µl of the DNA extract and 9 µl of sterile distilled water in a final volume of 25 µl. The thermal cycling was carried out according to the following protocol: 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C and a final extension of 7 min at 72°C, followed by cooling at 4°C.

Capillary electrophoresis

The capillary electrophoresis apparatus used was the ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, Ca.). Electrophoresis was done in a single capillary, filled with liquid polymer (POP-4: Performance Optimized Polymer, Applied Biosystems), which was automatically replaced after every electrophoresis run. Preparation of the sample before electrophoresis consisted of adding 1 µl of ITS2-PCR product to 12.5 µl of electrophoresis mixture (0.1 µl HD 400 marker, 0.3 µl of ROX-500 marker and 12.1 µl of deionized formamide, all from Applied Biosystems). The double stranded ITS2-PCR fragments were denatured by heating this mixture for 2 minutes at 95°C, followed by immediate cooling on ice. Electrophoresis of each sample was carried out at 60°C and at 15 kV, during 35 min. The Gene Scan analysis software (Applied Biosystems) was used to derive the fragment length of the HEX-labeled DNA-fragments using the known fragment lengths of the ROX-labeled marker peaks. The results were presented in a table indicating length and intensity of the observed fragments. BaseHopper software, described previously [12,13] and freely available upon request was used to quickly compare the obtained ITS2-fragment lengths with those of the ITS2-database.

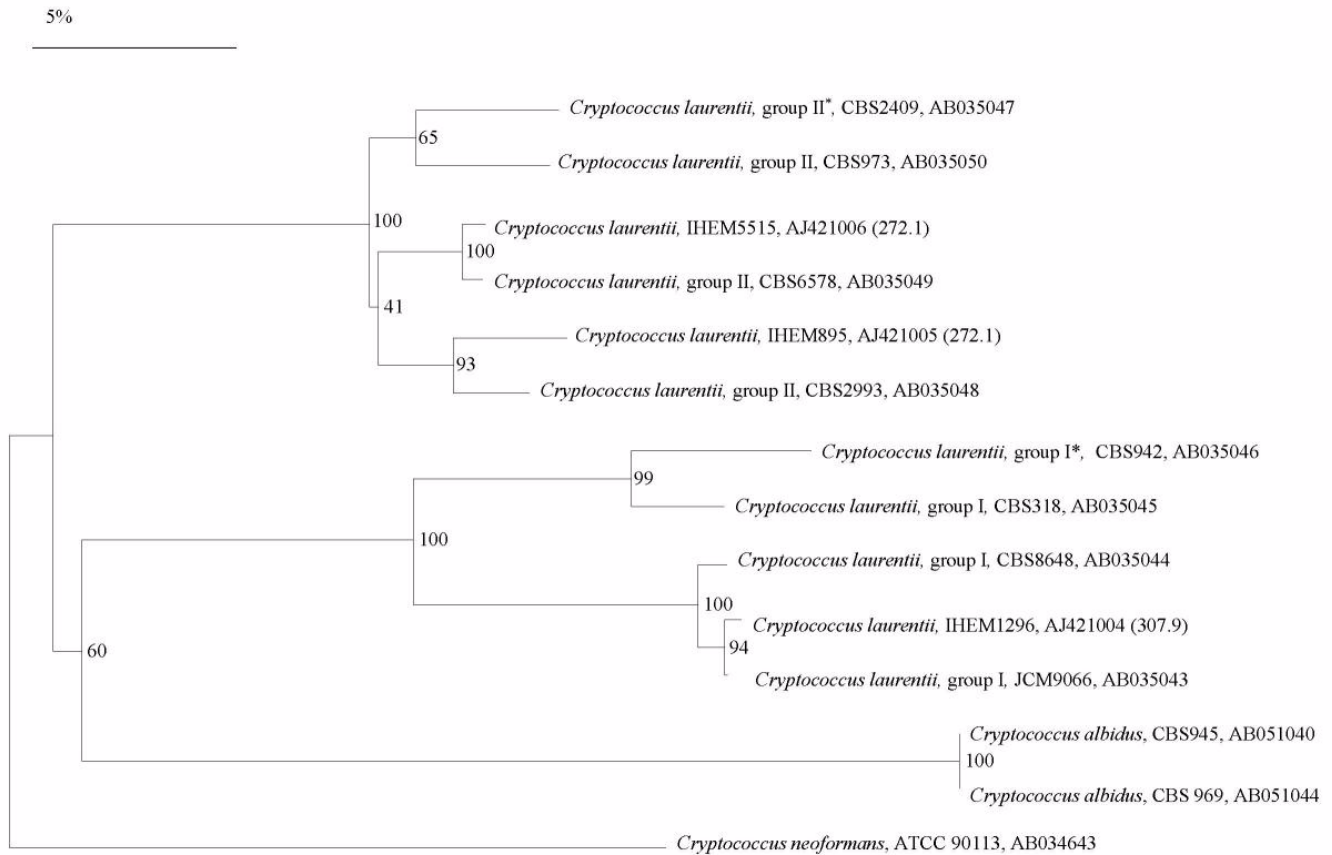


Figure 2
Dendrogram of ITS2 sequences of *Cryptococcus laurentii*, *C. albidus* and *C. neoformans*. Numbers on branches indicate bootstrap value. Species name, phylogenetic group according to Sugita et al.[11], strain number, GenBank accession number (and ITS2-length in bp) are indicated.

Sequencing of the ITS2 region

After amplification of the ITS2 region, the amplicon was purified using the Qiaquick PCR purification kit, according to the manufacturer's instructions. Cycle sequencing was performed using the Ready-reaction mix (ABI Prism Bigdye Terminator Cycle Sequencing kit, Applied Biosystems) according to the manufacturer's instructions. Analysis of the sequencing products was done on the ABI Prism 310 capillary (Applied Biosystems). Assembly of sequence fragments and editing was done with GeneBase (Applied Maths, St. Martens Latem, Belgium). The obtained ITS2 sequences were compared to all known sequences in the Genbank by use of Blast 2.0 (National Center for Biotechnology Information, Bethesda, Md. [http://www.ncbi.nlm.nih.gov/BLAST/]).

Cluster analysis and dendrogram construction of ITS2 sequences

Comparative sequence analysis was performed by using the GeneBase software package (Applied Maths, St. Martens Latem, Belgium), as described previously [15]. First, pair wise alignment using UPGMA was carried out with a gap penalty of 100 %, a unit gap cost of 20 % and an ambiguity cost of 50 % of the mismatch cost. Subsequently, global alignment – with *C. neoformans* ATCC 90113 (Genbank AB034643) used as the outgroup – was carried out on the region between 26 and 205 bp of the ITS2 region of *C. neoformans*, with costs as above. Finally, a similarity matrix of the aligned sequences was constructed by global alignment homology calculation and a gap penalty of 20 %. The neighbour-joining method was used to construct the dendrogram based on this similarity matrix. Bootstrap (n=100) values were calculated.

Authors' contributions

Authors 1 (TD), 4 (CM) and 6 (AM) carried out the molecular studies and participated in the sequencing work and the interlaboratory comparisons. Authors 2 (GC), 3 (DS) and 5 (GV) provided the clinical and reference strains, Authors 1 (TD), 2 (GC), 5 (GV) and 7 (MV) participated in the design of the study and author 7 (MV) coordinated the study and drafted the manuscript.

All authors read and approved the final manuscript.

Abbreviations

alb: *Candida albicans*

dub: *C. dubliniensis*

gla m: *C. glabrata*, minor peak

gla M: *C. glabrata*, major peak

gui: *C. guilliermondii*

lip: *C. lipolytica*

lus: *C. lusitaniae*

rug: *C. rugosa*

par: *C. parapsilosis*

nor: *C. norvegensis*

tro: *C. tropicalis*

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V. Application of PCR-based identification techniques for classification and description of newly or infrequently encountered species.

V.1. Introduction: Bacterial systematics.

Systematics is the scientific study of organism diversity. It is a fundamental discipline that encompasses classification, nomenclature and identification. Classification is the ordering of organisms into groups (taxa). Nomenclature is the means by which organisms are allocated internationally recognised scientific names. Identification is the means by which unknown organisms are placed into previously described taxa, derived from classification (Priest and Austin, 1993).

There are three good reasons to classify organisms: (i) classification is an efficient means of summarising and cataloguing information about an organism; (ii) databases of grouped organisms allow new isolates to be catalogued and ordered into their appropriate taxa, thereby facilitating identification and (iii) insight can be gained into the origins and evolutionary history of organisms.

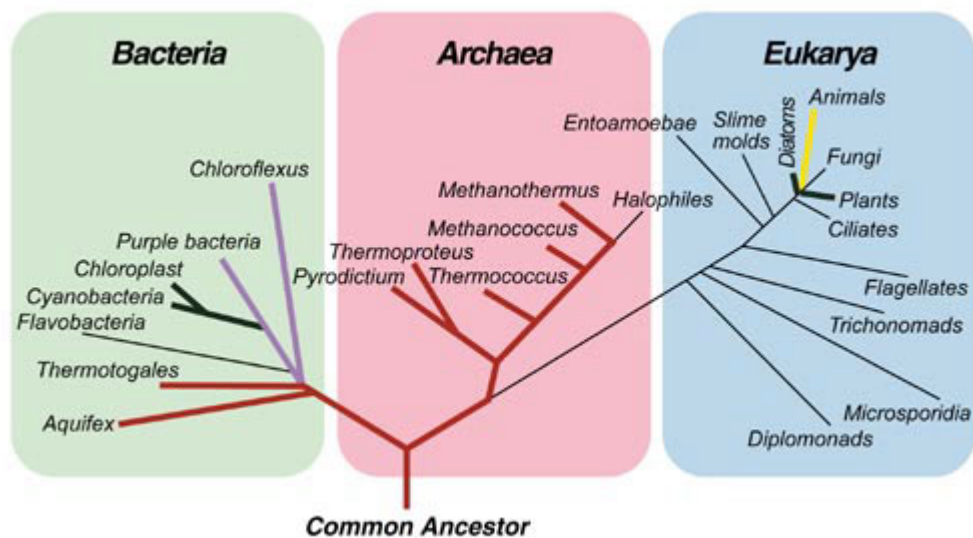
Using structural and phenotypical properties of organisms, all life on Earth has for a long time been classified into five Kingdoms: Plantae, Animalia, Protista, Prokaryota and Fungi. However, currently only three Domains are being recognized: Bacteria, Archae and Eukarya (Woese *et al.*, 1990) (Figure 5).

The most useful molecules for phylogenetic measurement are those that are essential to basic functioning and are therefore likely to span the full evolutionary spectrum. One such family of molecules are the ribosomal RNA genes. Ribosomal RNAs are essential elements in protein synthesis, and are therefore conserved in all living organisms. They have changed very little over time and therefore even the most taxonomically distinct organisms will share a degree of rRNA homology and therefore relatedness can be assessed. Certain regions of the rRNA gene evolve more rapidly than others and sequence variation occurs between closely related bacteria, allowing comparisons to be made at the species level.

The amount of homology between rRNA sequences of organisms will reflect phylogenetic relatedness. High homology implies recent divergence from a common ancestor and low homology implies early divergence from a primitive ancestor (Priest and Austin, 1993; Woese, 1987).

This knowledge leads towards the classification based on the small subunit rRNA (16S or 18S rRNA gene), which revealed that cellular life on Earth has evolved along three major lineages. Two of them had remained exclusively microbial and are composed only of prokaryotic cells and are called Bacteria and Archaea. The third line consists of the eucaryotic lineage, called Eukarya to which the Fungi belong (Figure 5).

Figure 5: Phylogenetic tree of life, based on the small subunit rRNA homologies



The introduction of the small ribosomal subunit sequence as basis for phylogeny and classification enabled the use 16S rRNA gene sequencing for identification of unknown isolates and for classification of newly encountered species in relation to the already known species. Sequencing of 16S rRNA genes resulted in a characteristic that could be compared and classified and expressed as a percentage of similarity. It has been proposed that a prokaryote whose 16S rRNA sequence differs by more than 3% from that of all other organisms, should be considered as a new species (Stackebrandt & Goebel 1994).

There are, however, some important drawbacks in the use of 16S rRNA gene sequencing for phylogeny.

(i) The homology rate between members of certain genera (e.g. *Staphylococcus*, *Bacillus*, *Aeromonas*, *Enterococcus*) is too high, such that the 3% rule is not applicable. For example, it

was found that on the basis of 16S rRNA sequence data, all *Bacillus* species should be considered as a single species, having 99.5% sequence identity (Fox *et al.*, 1992). Previously published DNA-DNA relatedness data contradicted this result;

(ii) The presence of mosaic rRNA genes, which indicates the occurrence of horizontal transfer and recombination of (parts of) rRNA cistrons between strains and species (Sneath, 1993; Gürtler, 1999);

(iii) Heterogeneity among the different alleles of the 16S rRNA genes (one to ten copies) (Gürtler and Stanisch, 1996; Rainey *et al.*, 1994), which can sometimes be very high, up to 5% in certain strains (Vanechoutte and Heyndricks, 2000).

Due to those several limitations 16S rRNA gene sequencing can not be used as the sole classification method. In practice, the identification commonly implies a polyphasic approach using several characteristics, and classification is based on a polyphasic taxonomy (Vandamme *et al.*, 1996).

An *ad hoc* committee on reconciliation of approaches to bacterial systematics in 1987 stated that the reference standard by which phylogeny is determined is complete DNA sequence data; and that phylogeny should also determine taxonomy. The committee members stated that the species is the only taxonomic unit that can be defined in taxonomic terms and they proposed a polyphasic approach to its definition, in which a DNA reassociation value of about 70% plays a dominant role (Wayne *et al.*, 1987; Stackebrandt and Goebel, 1994). An update of the recommendations on species definition is currently published (Stackebrandt *et al.*, 2002).

V.2. Overview of the taxonomy of the genera studied.

Identification of clinical isolates, making use of the molecular identification techniques presented in chapter IV, can reveal unsuspected pathogens amongst the majority of clinical suspected with known clinical importance or strains difficult to place on the exact taxonomical positions. These isolates are the basis for further taxonomical research leading to the description of new species, or case reports.

During this doctoral work, taxonomical studies were carried out on *Acinetobacter* and *Mycobacterium* using ARDRA and additional techniques like biochemistry, 16S rRNA gene sequencing and DNA-DNA hybridizations. This has resulted in the description of two new species in the genus *Acinetobacter* and the review of the clinical importance of *M. interjectum* after such an isolate was seen in our hospital. Other genera of interest were *Ralstonia*, leading to the description of *R. mannitolilytica* and the publication of two case reports with *R. gilardii* and *R. mannitolilytica* isolates. Finally the genus *Moraxella* was studied because of the two case reports of clinical isolations of *M. atlantae* and *M. canis*.

These publications are included and are introduced by a taxonomical overview of the genera of interest.

V.2.1. Taxonomy of the genus *Acinetobacter*.

Bacteria of the genus *Acinetobacter* (Brisou and Prévot, 1954) are widespread in nature, and can be recovered from water, soil and living organisms. They are non-motile, non-fermentative, coccobacillary, strictly aerobic and Gram-negative. They can use a wide variety of different carbon sources for growth, and can be cultured on simple media. They are oxidase-negative and catalase-positive. Their mol% G+C of the DNA is 38-47. The genus is placed within the gamma-subclass of the *Proteobacteria* (Railey *et al.*, 1994).

The type species of the genus is *Acinetobacter calcoaceticus*.

The first classification was presented by Bouvet & Grimont (1986) who distinguished 12 different groups using DNA-DNA hybridization. Seven of these received a validated species name. Three years later (Tjernberg and Ursing, 1989) three additional DNA groups were described, coded 13 through 15 TU. In the same year another group (Bouvet and Jeanjean, 1989) described five additional proteolytic *Acinetobacter* species, numbered 13 through 17. The DNA group 13 BJ appeared to correspond to 14 TU (Vaneechoutte *et al.*, 1995), while for the other groups no correlation is apparent. Apart from the known genomic species,

additional strains were found, some of which are closely related to already known groups, like the DNA group CTTU 13: “close to Tjernberg-Ursing DNA group 13”, 1-3L: “genospecies 1 or 3-like”, and genospecies “between 1 and 13”. (Gerner-Smidt and Tjernberg, 1998).

A. baumannii, and the unnamed groups 3 and 13 TU are recovered predominantly from clinical specimens, with *A. baumannii* being known for its capacity to colonize and infect severely ill, hospitalised patients. Strains of this genomic species can persist in hospitals and give rise to outbreaks. They are usually highly resistant to antibiotics, which makes them difficult to eradicate. The other species are more known as environmental isolates, and only rarely encountered as clinical isolates.

Using ARDRA, it could be shown that the well known industrially important, emulsan-producing strain RAG-1, isolated from tar on a beach in Israel (Rosenberg *et al.*, 1979), an isolate designated ‘*A. venetianus*’, found in the lagoon of Venice, capable of oil-degradation (Di Cello *et al.*, 1997), and a n-tetradecane-degrading marine strain (T4) (Yamamoto and Harayama, 1996) belonged to the same species (Vanechoutte *et al.*, 1999).

By sequencing the 16S rRNA genes, in addition to ARDRA and phenotypic data, it was possible to contribute to the description of two new species, *A. ursingii* and *A. schindleri*, of which several strains have clinical importance (Nemec *et al.*, 2001).

Currently, the genus *Acinetobacter* comprises at least 23 genomic species (DNA-DNA hybridisation groups, DNA groups), 10 of which have been given species names. Table 3 represents an overview of all the species and DNA-groups.

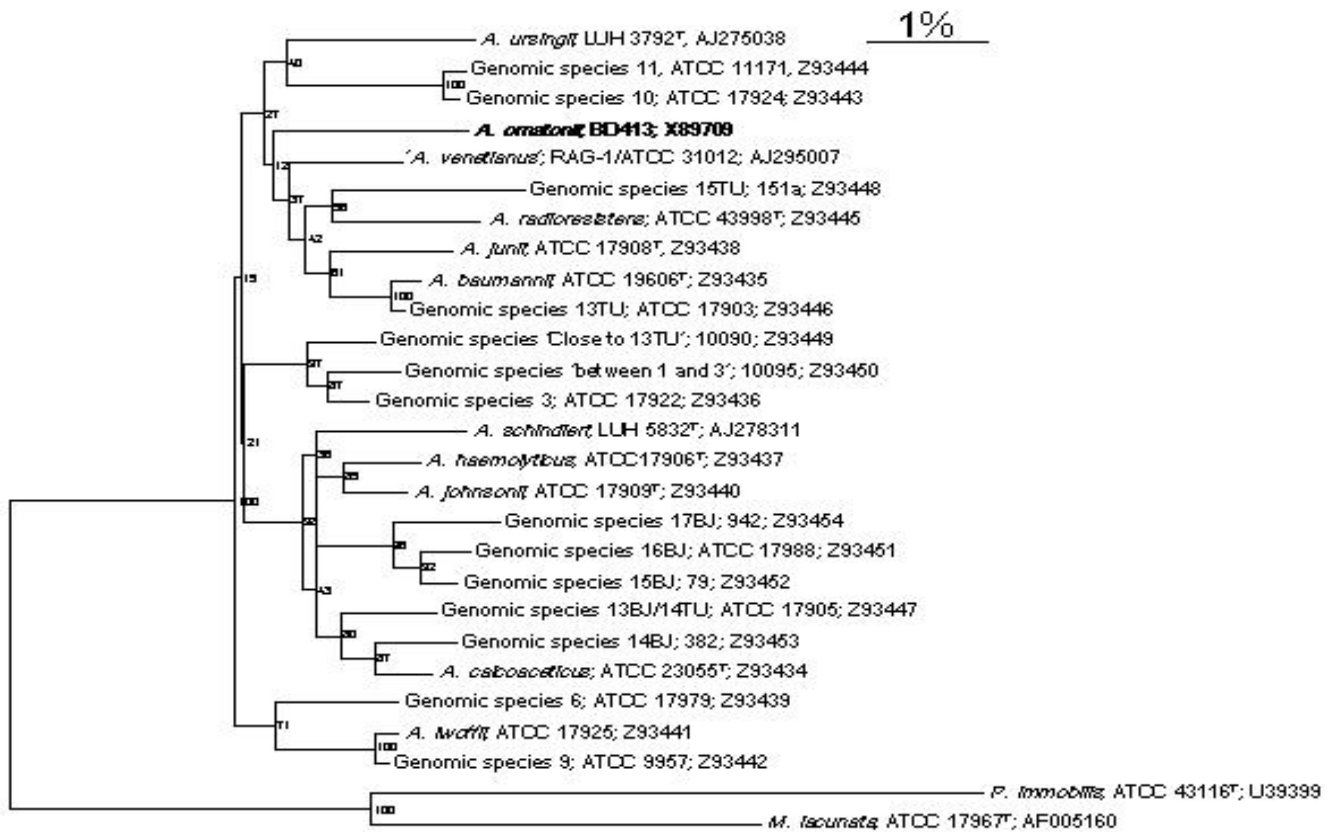
Table 3: Overview of the *Acinetobacter* DNA-groups and corresponding nomenspecies.

DNA-Group	Species	Reference
1	<i>A. calcoaceticus</i>	Bouvet and Grimont, 1986
2	<i>A. baumannii</i>	Bouvet and Grimont, 1986
3		Bouvet and Grimont, 1986
4	<i>A. haemolyticus</i>	Bouvet and Grimont, 1986
5	<i>A. junii</i>	Bouvet and Grimont, 1986
6		Bouvet and Grimont, 1986
7	<i>A. johnsonii</i>	Bouvet and Grimont, 1986
8/9*	<i>A. lwoffii</i>	Bouvet and Grimont, 1986
10		Bouvet and Grimont, 1986
11		Bouvet and Grimont, 1986
12	<i>A. radioresistens</i>	Nishimura <i>et al.</i> , 1988
13TU		Tjernberg and Ursing, 1989
13 BJ / 14 TU		Bouvet and Jeanjean, 1989 Tjernberg and Ursing, 1989
14 BJ		Bouvet and Jeanjean, 1989
15 BJ		Bouvet and Jeanjean, 1989
15 TU		Tjernberg and Ursing, 1989
16 BJ		Bouvet and Jeanjean, 1989
17 BJ		Bouvet and Jeanjean, 1989
	<i>A. venetianus</i>	Di Cello <i>et al.</i> , 1997, Vanechoutte <i>et al.</i> , 1999
	<i>A. schindleri</i>	Nemec <i>et al.</i> , 2001
	<i>A. ursingii</i>	Nemec <i>et al.</i> , 2001

* DNA groups 8 and 9 were later shown to be synonymous (Tjernberg and Ursing, 1989).

Figure 6 represents a similarity tree based on 16S rRNA gene sequences of most of the ARDRA groups and phenological groups currently known, besides the 23 DNA-groups.

Figure 6: Similarity tree based on 16S rRNA gene sequence similarities



Legend: Neighbour-Joining phylogenetic tree, based on 16S rRNA gene sequences comparison, calculating the global alignment similarities on a region corresponding to *E. coli* positions 67-1444. Global alignment was carried out with a gap penalty of 100%, a unit gap penalty of 20% and an ambiguity cost of 50%. Boot strap values (n=100) are indicated at the nodes, and the scale bar is 1% sequence dissimilarity (one substitution on 100 nucleotides).

Bacteria can be identified to the genus *Acinetobacter* by a list of the phenotypic criteria. However, a simple test is based on the finding that DNA of organisms belonging to the genus can be used to transform an auxotrophic *Acinetobacter* strain (BD413) to prototrophy (Juni, 1972). According to the 16S rRNA gene sequence of this strain, as obtained during this doctoral work, strain BD413 belong to a separate species, indicated as '*A. ornstonii*' (Figure 6). During this work groups of clinical encountered species were identified as belonging to *Acinetobacter* species not yet published, further research is in progress towards the description of these species. One species of particular clinical importance, but overlooked until now, is phenon 4, for which description as *A. parvus* is in progress. The isolates are

biochemically inert and form very small nonhemolytic colonies, atypical for *Acinetobacter* (Nemec *et al.*, in progress).

Biochemical and phenotypical discrimination between the different species is difficult and needs a wide variety of different tests (Gerner Smith *et al.*, 1991; Kämpfer *et al.*, 1993).

Commercial systems like API and Biolog have only a moderate performance for the identification of *Acinetobacter* species (Bernards *et al.*, 1995; Bernards *et al.*, 1996).

Genotypical identification offers more possibilities. Different systems have been applied:

Ribotyping (Gerner-Smith, 1992), AFLP (Janssen *et al.*, 1997), ARDRA (Vanechoutte *et al.*, 1995; Dijkshoorn *et al.*, 1998), 16S rRNA gene sequencing (Ibrahim *et al.*, 1997), tRNA fingerprinting (Ehrenstein and Schön, 1996) and ribosomal spacer amplification (Nowak *et al.*, 1996). An overview of the performances of all these techniques is presented in Table 4.

Table 4: Comparison of the genotypical identification techniques for the members of the genus *Acinetobacter*.

Technique	Reference	Species used on publication	Sufficient number of strain/species	Validation		Speed or Practical applicability
				Discriminatory power	Interlaboratory	
Ribotyping	Gerner-Smith <i>et al.</i> , 1992	<i>A. calcoaceticus-baumannii</i> complex	+	<i>A. calcoaceticus-baumannii</i> complex	no	weak
Ribosomal spacer amplification	Nowak <i>et al.</i> , 1996	17 genospecies	+	complete genus	no	high
tRNA fingerprinting	Ehrenstein and Schön, 1996	all at that moment	+	all species, except for groups 1 vs 3 and 2 vs 13CTTU	no	high
AFLP	Janssen <i>et al.</i> , 1997	all at that moment	+	complete genus	no	weak
ARDRA	Dijkshoorn <i>et al.</i> , 1998	all at that moment	+	complete genus	yes	high
16S rRNA sequencing	Ibrahim <i>et al.</i> , 1997	all at that moment	±	complete genus	no, but accepted to be high	weak

V.2.2. Taxonomy of the genus *Moraxella*.

The genus *Moraxella*, is classified in the family of the *Moraxellaceae* (Rossau *et al.*, 1991), together with *Acinetobacter* and *Psychrobacter*. They were formerly known as ‘false neisseriae’.

The *Moraxella* are Gram-negative, non-motile aerobic species. Some of them have the ability for ‘twitching motility’ and spread on the culture plate. No flagella are observed and only occasionally some fimbriae. They are oxidase-positive, usually catalase-positive and no acid is produced from carbohydrates. The mol% G+C of the DNA ranges from 40 to 47,5.

A subdivision is sometimes made based on morphology whereby the rod-shaped species are classified in *Moraxella* (subgen. *Moraxella*) and the cocci in *Moraxella* (subgen. *Branhamella*) (Bøvre, 1984).

The genus currently consists of 15 validated species, which are summarized in Table 5.

Table 5: overview of the *Moraxella* species.

Species	Reference
<i>M. atlantae</i>	Bøvre <i>et al.</i> , 1976
<i>M. boevrei</i>	Kodjo <i>et al.</i> , 1997.
<i>M. bovis</i>	Bøvre <i>et al.</i> , 1984
<i>M. catarrhalis</i> *	Bøvre <i>et al.</i> , 1984
<i>M. caviae</i> *	Henriksen and Bøvre, 1968
<i>M. ovis</i> *	Bøvre <i>et al.</i> , 1984
<i>M. canis</i> *	Jannes <i>et al.</i> , 1993
<i>M. caprae</i> *	Kodjo <i>et al.</i> , 1997.
<i>M. cuniculi</i> *	Bøvre and Hagen, 1984
<i>M. equi</i>	Hughes and Pugh, 1970
<i>M. lacunata</i>	Lwoff, 1939
<i>M. lincolniai</i>	Vandamme <i>et al.</i> , 1993
<i>M. nonliquefasciens</i>	Bøvre <i>et al.</i> , 1984
<i>M. osloensis</i>	Bøvre <i>et al.</i> , 1984
<i>M. saccharolytica</i>	Flamm, 1956

* Species with coccal cell morphology (sometimes classified in the subgenus *Branhamella* (Bøvre *et al.*, 1984)

Two species, previously known as *Moraxella*, were placed in other genera during the last years: *M. urethralis* was changed to *Oligella urethralis* (Rossau *et al.*, 1987) and *M. phenylpyruvica* to *Psychrobacter phenylpyruvica* (Bowman *et al.*, 1996).

Suggestions for a separate classification of *M. osloensis*, *M. atlantae* and *M. lincolnii* were made based on 16S rRNA gene similarities (Pettersen *et al.*, 1998; De Baere *et al.*, 2002); DNA-DNA hybridisation (Tønjum *et al.*, 1989) and DNA-transformation (Bøvre *et al.*, 1976).

The clinically most important species is *Moraxella catarrhalis*, a human pathogen mainly known for causing upper airway infections. Some of the other species are known as pathogenic for animals, for example *M. bovis* (Chandler *et al.*, 1980).

During this doctoral work we described the first known human infection with *M. canis* (Vanechoutte *et al.*, 2001) and an infection with another rare pathogen, *M. atlantae* (De Baere *et al.*, 2002).

V.2.3. Taxonomy of the genus *Mycobacterium*.

The genus *Mycobacterium* is one of the oldest defined (1896) and the generic name *Mycobacterium* initially designated a group of organisms that grew as mould-like pellicules on liquid media (Lehman, 1896).

During the 1900s the characteristics used to define mycobacteria were the absence of motility, morphology of the bacilli (slightly curved and rod-shaped) and the characteristic resistance to acid-alcohol following coloration with phenic acid fuchsin (Ziehl-Neelsen stain), called acid-fastness.

Taxonomically *Mycobacterium* is the only genus in the family *Mycobacteriaceae*, which is part of the order of the *Actinomycetales*. Related taxa are *Corynebacterium*, *Nocardia*, *Rhodococcus*, *Gordona* and *Tsukamurella*.

Mycobacterium species and the members of the mentioned related genera are the only microbial organisms that are able to synthesize mycolic acids (Goodfellow and Wayne, 1982). Discrimination between those organisms can be done by Ziehl-Neelsen staining, the G + C base ratio, and the mycolic acid content (Vincent, 1994).

At present, the mycobacteria are defined as aerobic, acid-alcohol fast, rod-shaped actinomycetes with occasional branching: aerial hyphae are normally absent, and the bacteria are non-motile, non-sporulating organisms that contain arabinose, galactose, and meso-diaminopimelic in the cell wall. The GC base ratio ranges from 62 to 70 % (except for *M. leprae*: 58%). Mycolic acids of high molecular weight (sixty to ninety carbon atoms) are present, lacking components with more than two points of unsaturation in the molecule (Goodfellow and Wayne, 1982).

The most important member of the genus is the species *Mycobacterium tuberculosis*. This omnipresent organism is one of the most important and oldest known pathogens of the world, causing tuberculosis. Tuberculosis is known to have been present since antiquity (Rastoghi *et al.*, 2001) and it is suspected as the cause of death of some of the Egyptian mummies 3000 years ago, and has been proved as the cause of death, by evidence of pulmonary tuberculosis and the presence of acid-fast organisms, of a mummy of Peru, dated 700 AD.

From the 19th century, onward it was known as the 'White Plague' being the leading cause of death in Europe and the United States of America (4/1000 in the USA in 1830). Due to improvements in social and sanitary conditions the tuberculosis rate was dropped to 2/1000 in 1900. During the twentieth century major advances were seen in the struggle against

tuberculosis. At first there was the development of chemotherapy, based on streptomycine (1944), para-amino salicylic acid (1949), isoniazide (1952) and rifampicin (1967). On the other hand there was the vaccination program with the BCG strain (Bacillus Calmette-Guérin), used for the first time in 1921. This and the availability of good laboratory methods and still improving living conditions have lead to a steady decline of the tuberculosis incidence in the industrial countries (less than 1/10.000 in 1980 (Kalkut, 2000)). But the struggle has not (yet?) come to an end, because two major threats are the cause of a re-emergence of the deadly pathogen: co-infection with HIV (Pozniak, 2002) and the existence of multi-drug resistant strains (Espinal *et al.*, 2001).

The mycobacterial species are for practical reasons mostly divided in two groups: *M. tuberculosis* complex {*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and the recently described species *M. canettii* (Pfyffer *et al.*, 1998)} and ‘mycobacteria other than the *M. tuberculosis* complex’ or MOTT, sometimes called atypical mycobacteria.

MOTTs have been subdivided in four different groups. (Runyon, 1959)

Group I: the photochromogenic species, whereby the colonies acquire pigmentation in the presence of light only (*M. kansasii*, *M. marinum*, ..).

Group II: the scotochromogenic species whereby the colonies acquire pigmentation in the presence or absence of light. (*M. gordonae*, *M. scrofulaceum*, ...)

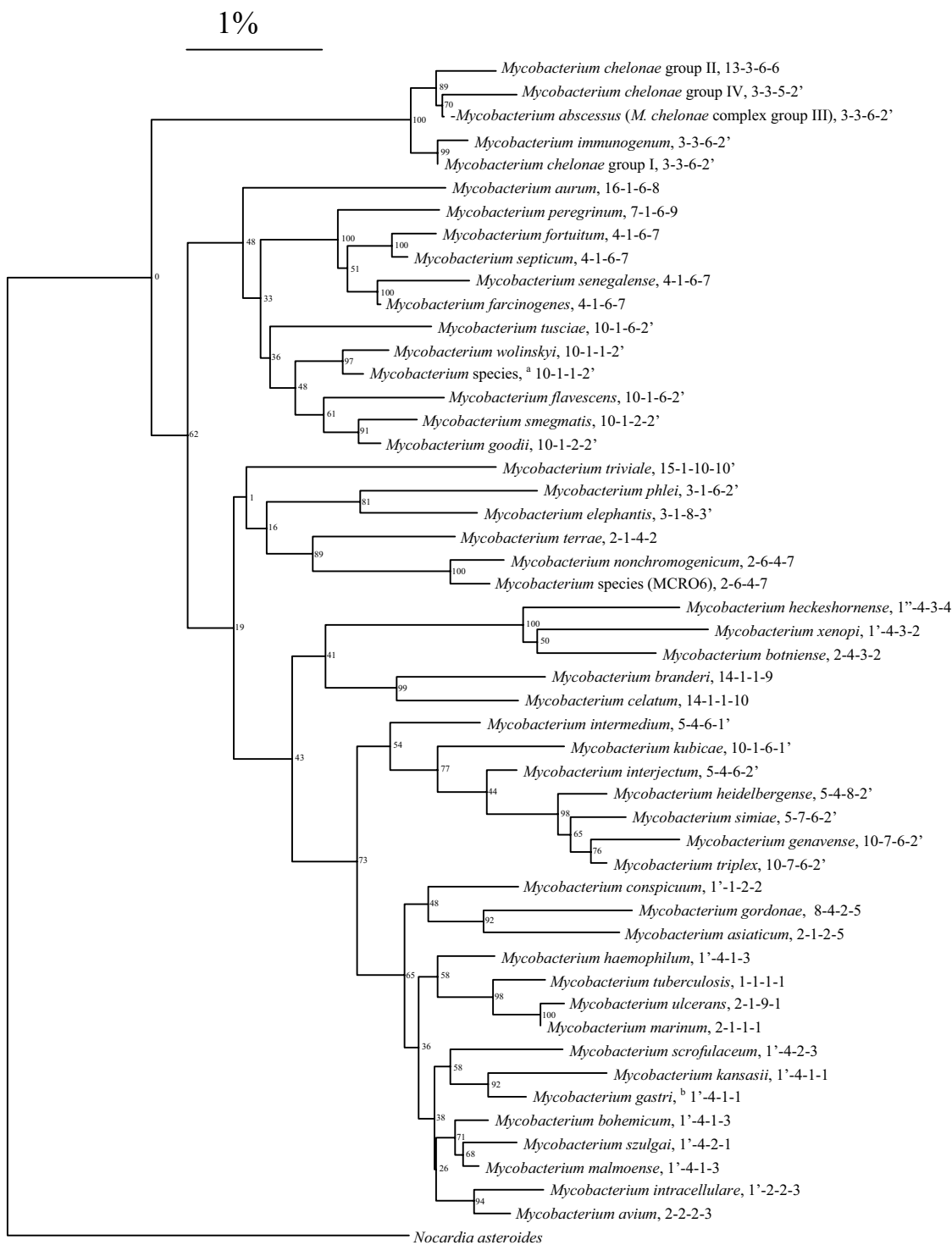
Group III: non-chromogenic species which have non-pigmented colonies (*M. avium*, *M. intracellulare*,...)

Group IV: rapid growers (*M. fortuitum*, *M. chelonae*).

Slow growers (Groups I to III) have a mean division time of 12 to 24 hours, and a fully-grown culture requires approximately fifteenth till twenty-eight days. The average division time for fast growers is 2 to 6 hours, with a culture available within two to seven days.

The taxonomy of *Mycobacterium* has become very complex, with almost 100 species described, 19 of them reported during the last 5 years. An overview of the species, containing amongst others, the clinical important species; but restricted to those that have 16S rRNA gene sequences available in Genbank, is presented in Figure 7.

Figure 7: A phylogenetic tree of representants of the genus *Mycobacterium*.



Legend: Neighbour-Joining phylogenetic tree of the 16S rRNA genes of the clinically important *Mycobacterium* species, with the addition of their ARDRA pattern. (De Baere *et al.*, 2002).

A wide variety of different technologies are used for the differentiation of *Mycobacterium* species. Some well-known techniques were evaluated recently in literature: HPLC analysis of mycolic acids (Butler and Guthertz, 2001); hsp65 fingerprinting PCR (Brunello *et al.*, 2001), 16S rRNA gene sequencing (Turenne *et al.*, 2001) and a commercial probe test Inno-Lipa Mycobacteria (Suffys *et al.*, 2001).

During this doctoral thesis the PCR-based DNA-fingerprinting technique ARDRA was updated and evaluated for the identification of *Mycobacterium* species (De Baere *et al.*, 2002).

Using ARDRA, an isolate from a lymph node of a child with cervical lymphadenitis was identified as *M. interjectum*. Analysis of this case and previously published cases indicated the importance of *M. interjectum* as a causative agent of cervical lymphadenitis in young children (De Baere *et al.*, 2001).

V.2.4. Taxonomy of the genus *Ralstonia*.

The genus *Ralstonia* (Yabuuchi *et al.*, 1995) has been created for a group of organisms from ecologically diverse niches to accommodate bacteria that were formerly classified as members of *Burkholderia* (Yabuuchi *et al.*, 1992) and *Alcaligenes*. The type species of the genus is *Ralstonia pickettii* (Ralston *et al.*, 1973).

They are Gram-negative rod-shaped cells, which are either motile with a single flagellum (polar or peritrichous) or non-motile without flagella. Monosaccharides, disaccharides and polyalcohols are oxidized and assimilated as a sole source of carbon and energy. The GC content is around 65 mol%, with *Burkholderia* as the closest genus. *Ralstonia* species, in contrast to *Burkholderia* species, are negative for assimilation of galactose, mannitol and sorbitol. The description of *R. mannitolilytica* (De Baere *et al.* 2001) showed however that mannitol assimilation is not a discriminating factor anymore, because this formerly known subspecies of *R. picketti* assimilates mannitol.

The type species *R. pickettii* was originally regarded as the only representative of clinical importance (Gardner and Shulman, 1984; Lacey and Want, 1991; Raveh *et al.*, 1993; Verschraegen *et al.*, 1985). The other members in the first description were *Ralstonia solanacearum*, one of the most important bacterial phytopathogenic species, causing bacterial wilt on a wide range of crops (Palleroni and Doudoroff, 1971) and *R. eutropha*, an environmentally encountered bacterium (Davis, 1969) (Figure 6a).

Only recently, the taxonomy of this genus has begun to be elucidated with the description of several new species. In 1998 the first new member was *R. basilensis* (Steinle *et al.*, 1998), a species isolated from a fresh water pond and able to degrade 2,6 dichloro-phenol. The following years the species *R. gilardii* (Coenye *et al.*, 1999) and *R. paucula* (Vandamme *et al.*, 1999) were added. These species are environmental and sporadic cases of infection with clinical evidence have been described (Anderson *et al.*, 1997; Wauters *et al.*, 2001).

During this doctoral work we described the formerly known third biovar of *R. pickettii* as the separate species *R. mannitolilytica* (De Baere *et al.*, 2001) and reported two clinical cases (Vaneechoutte *et al.*, 2001). Two metal-resistant bacteria, *R. campinensis* and *R. metallidurans* (Goris *et al.*, 2001) and a plant pathogen *R. taiwanensis* (Chen *et al.*, 2001) were also added (Figure 6b).

Most of the new findings were based on 16S rRNA sequence data, corroborated by phenotypic testing (Prof. Em. Dr. G. Wauters). tRNA-PCR did not appear to be suited for *Ralstonia* species identification, since most species gave no or only few amplification fragments.

16S rRNA gene sequencing and phenotypic testing led to the description of *R. mannitolilytica* and to a proposal to split the genus *Ralstonia* into *Ralstonia sensu stricto* and *Wautersia* gen. nov. (Vaneechoutte *et al.*, in preparation). Indeed the newly obtained sequence data in this doctoral study, together with those available from the Genbank showed a clear bifurcation within the genus (Fig 6c). This division corresponded with clear morphological and biochemical differences between both groups, as observed by Prof. Em. Dr. G. Wauters, with the flagellation as the most important discriminating factor.

Figure 8: The changing taxonomy of the genus *Ralstonia* during history, based on 16S rRNA gene sequence similarity.

Figure 8a. Situation in 1995 (Yabuuchi *et al.*, 1995)

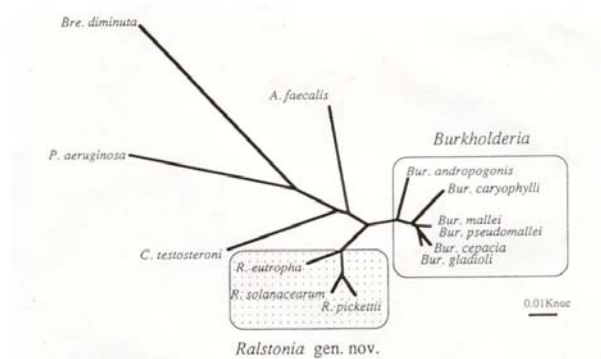


Figure 8b. Current situation (Chen *et al.*, 2001)

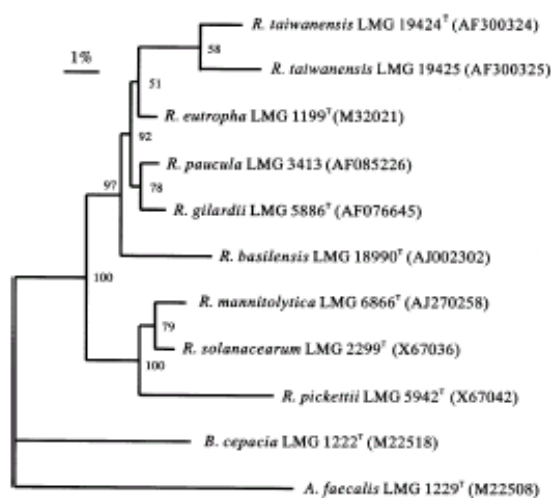
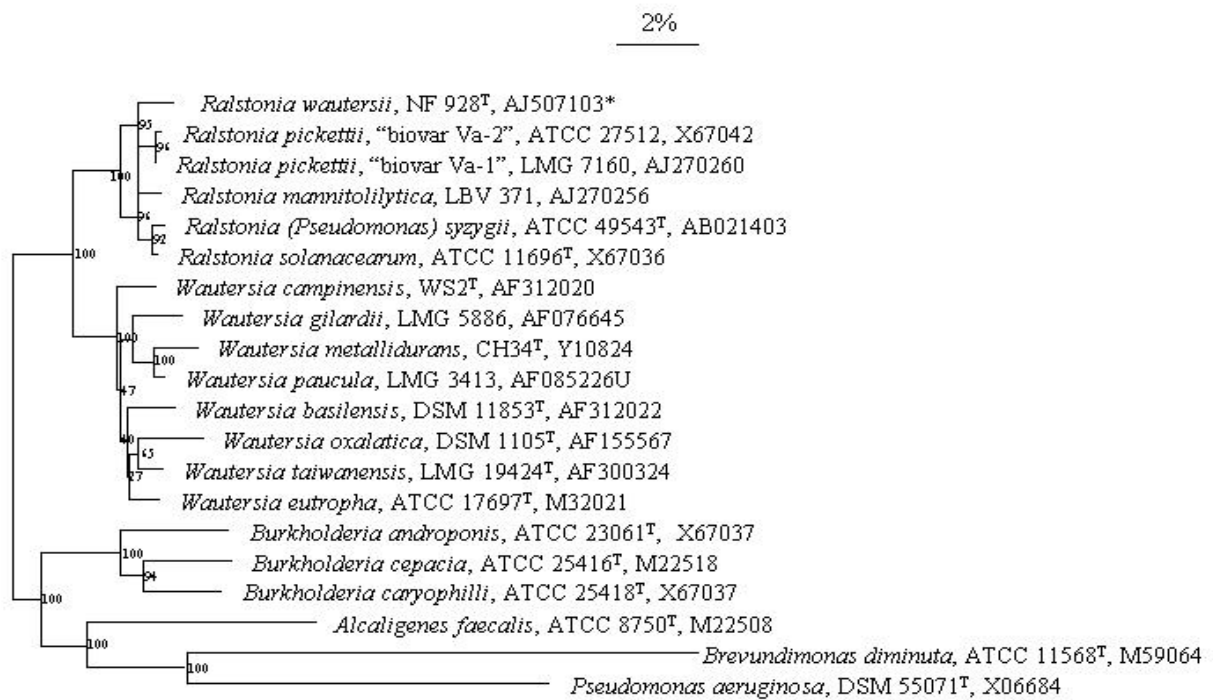


Figure 8c: The proposed taxonomy for the near future (Vanechoutte *et al.*, in preparation)



V.3. Studies

The described DNA-based fingerprinting techniques (IV.3) could be used for precise identification of different kinds of organisms. Their application on large series of isolates revealed species not yet described before (V.3.1. and V.3.5) and species infrequently encountered in the clinical setting (V.3.2.; V.3.3; V.3.4; V.3.6; V.3.7.).

V.3.1. Description of *Acinetobacter ursingii* and *A. schindleri*.

Nemec, A., T. De Baere, I. Tjernberg, M. Vaneechoutte, T.J.K. van der Reijden, and L. Dijkshoorn. 2001. *Acinetobacter ursingii* sp. nov. and *Acinetobacter schindleri* sp. nov., isolated from human clinical specimens. Int. J. Syst. Evol. Microbiol. **51**:1891-1899.

V.3.2. Ulcerated lymph node containing *Moraxella canis*.

Vaneechoutte, M., G. Claeys, S. Steyaert, T. De Baere, R. Peleman, and G. Verschraegen. 2000. *Moraxella canis* isolation from an ulcerated metastatic lymph node. J. Clin. Microbiol. **38**:3870-3871.

V.3.3. Bacteremia with *Moraxella atlantae*.

De Baere, T., A. Muylaert, E. Everaert, G. Wauters, G. Claeys, G. Verschraegen, and M. Vaneechoutte. 2002. Bacteremia with *Moraxella atlantae* in a cancer patient. J. Clin. Microbiol. **40**: 2693-2695.

V.3.4. *Mycobacterium interjectum* as a causative agent of lymphadenitis.

De Baere, T., M. Moerman, L. Rigouts, C. Dhooge, H. Vermeersch, G. Verschraegen, and M. Vaneechoutte. 2001. *Mycobacterium interjectum* as causative agent of cervical lymphadenitis. J. Clin. Microbiol. **39**:725-727.

V.3.5. Description of *Ralstonia mannitolilytica*

De Baere, T., S. Steyaert, G. Wauters, P. De Vos, J. Goris, T. Coenye, T. .Suyama, G. Verschraegen, and M. Vaneechoutte. 2001. Classification of *Ralstonia pickettii* biovar 3/'*thomasi*' strains (Pickett 1994) and of new isolates related to nosocomial recurrent meningitis as *Ralstonia mannitolilytica* sp. nov. Int. J. Syst. Evol. Microbiol. **51**:547-558

V.3.6. Two separate case reports of *Ralstonia mannitolilytica*

Vaneechoutte, M., T. De Baere, G. Wauters, S. Steyaert, G. Claeys, D. Vogelaers, and G. Verschraegen. 2001. One case each of recurrent meningitis and hemoperitoneum infection with *Ralstonia mannitolilytica*. J. Clin. Microbiol. **39**:4588-4590.

V.3.7. Catheter sepsis with *Ralstonia gilardii*

Wauters, G., G. Claeys, G. Verschraegen, T. De Baere, E. Vandecruys, L. Van Simaey, C. De Ganck, and M. Vaneechoutte. 2001. Case of catheter sepsis with *Ralstonia gilardii* in a child with acute lymphoblastic leukemia. J. Clin. Microbiol. **39**:4583-4584.

***Acinetobacter ursingii* sp. nov. and *Acinetobacter schindleri* sp. nov., isolated from human clinical specimens**

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The taxonomic status of two recently described phenetically distinctive groups within the genus *Acinetobacter*, designated phenon 1 and phenon 2, was investigated further. The study collection included 51 strains, mainly of clinical origin, from different European countries with properties of either phenon 1 (29 strains) or phenon 2 (22 strains). DNA–DNA hybridization studies and DNA polymorphism analysis by AFLP revealed that these phenons represented two new genomic species. Furthermore, 16S rRNA gene sequence analysis of three representatives of each phenon showed that they formed two distinct lineages within the genus *Acinetobacter*. The two phenons could be distinguished from each other and from all hitherto-described *Acinetobacter* (genomic) species by specific phenotypic features and amplified rDNA restriction analysis patterns. The names *Acinetobacter ursingii* sp. nov. (type strain LUH 3792^T = NIPH 137^T = LMG 19575^T = CNCTC 6735^T) and *Acinetobacter schindleri* sp. nov. (type strain LUH 5832^T = NIPH 1034^T = LMG 19576^T = CNCTC 6736^T) are proposed for phenon 1 and phenon 2, respectively. Clinical and epidemiological data indicate that *A. ursingii* has the capacity to cause bloodstream infections in hospitalized patients.

Keywords: *Acinetobacter ursingii* sp. nov., *Acinetobacter schindleri* sp. nov., polyphasic taxonomy

INTRODUCTION

Over the last 15 years, considerable progress has been made in resolving the taxonomy of the genus *Acinetobacter*. The basis for the present classification was established by Bouvet & Grimont (1986), with the description of 12 DNA–DNA hybridization groups (genomic species) within the genus. This scheme was subsequently extended to include 10 additional genomic species (Tjernberg & Ursing, 1989; Bouvet & Jeanjean, 1989; Gerner-Smidt & Tjernberg, 1993). Seven genomic species have names (*Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Acinetobacter haemolyticus*, *Acinetobacter junii*, *Acinetobacter john-*

sonii, *Acinetobacter lwoffii* and *Acinetobacter radio-resistens*), while the others are designated by numbers (reviewed by Janssen *et al.*, 1997). Another genomic species ('*Acinetobacter venetianus*') comprising marine oil-degrading organisms was delineated recently (Di Cello *et al.*, 1997; Vaneechoutte *et al.*, 1999). Nevertheless, the DNA–DNA hybridization studies of Bouvet & Grimont (1986), Tjernberg & Ursing (1989) and Bouvet & Jeanjean (1989) left several strains unclassified, which indicates that the diversity of the genus extends beyond the described groups.

In a recent study, 45 additional unidentifiable isolates were found among 700 clinical isolates from the Czech Republic (Nemeč *et al.*, 2000). Two groups of isolates (designated phenon 1 and phenon 2) were delineated among the unidentifiable isolates, each of which showed distinctive phenotypic features and amplified rDNA restriction analysis (ARDRA) patterns. The aim of the present study was to define the taxonomic

Abbreviations: ARDRA, amplified rDNA restriction analysis.

The EMBL accession numbers for the 16S rRNA gene sequences of strains LUH 3299, LUH 3792^T, LUH 4763, LUH 4591, LUH 4760 and LUH 5832^T are respectively AJ275037–AJ275041 and AJ278311.

Table 1. Strains of phenon 1 (*Acinetobacter ursingii* sp. nov.) and phenon 2 (*Acinetobacter schindleri* sp. nov.)

All strains were from human specimens. CNCTC, Czech National Collection of Type Cultures, Prague, Czech Republic; LMG, Bacteria Collection, Laboratorium voor Microbiologie Gent, Gent, Belgium; LUH and RUH, Collection L. Dijkshoorn, Leiden University Medical Centre, Leiden, The Netherlands; NIPH, Collection A. Nemeč, National Institute of Public Health, Prague, Czech Republic. Abbreviations: CZ, Czech Republic; NL, The Netherlands; NO, Norway; SE, Sweden.

Strain	Other strain designation(s)	Reference/received from	Specimen*	Location and year of isolation
Phenon 1 (<i>A. ursingii</i> sp. nov.)				
LUH 3792 [†]	NIPH 137 [†] = LMG 19575 [†] = CNCTC 6735 [†]	Nemeč <i>et al.</i> (2000)	Blood (in)	Praha, CZ, 1993
LUH 4582	NIPH 177 [†]	Nemeč <i>et al.</i> (2000)	Intravenous line (in)	Praha, CZ, 1993
LUH 4592	NIPH 280 [†]	Nemeč <i>et al.</i> (2000)	Blood (in)	Sedlčany, CZ, 1994
LUH 3793	NIPH 371 [†]	Nemeč <i>et al.</i> (2000)	Blood (in)	Příbram, CZ, 1995
LUH 4613	NIPH 375 [†]	Nemeč <i>et al.</i> (2000)	Pus (in)	Příbram, CZ, 1995
LUH 4614	NIPH 376 [†]	Nemeč <i>et al.</i> (2000)	Pus (in)	Příbram, CZ, 1995
LUH 4618	NIPH 398 [†]	Nemeč <i>et al.</i> (2000)	Ulcer (out)	Příbram, CZ, 1996
LUH 4622	NIPH 439 [†]	Nemeč <i>et al.</i> (2000)	Eye (out)	Sedlčany, CZ, 1996
LUH 5767	NIPH 706 [†]	Nemeč <i>et al.</i> (2000)	Blood (in)	Příbram, CZ, 1997
LUH 5829	NIPH 950 [†]	Nemeč <i>et al.</i> (2000)	Blood (in)	Tábor, CZ, 1998
LUH 5830	NIPH 993 [†]	Nemeč <i>et al.</i> (2000)	Cervix (out)	Příbram, CZ, 1998
LUH 5831	NIPH 1025 [†]	Nemeč <i>et al.</i> (2000)	Eye (out)	Příbram, CZ, 1998
LUH 5833	NIPH 1048 [†]	Nemeč <i>et al.</i> (2000)	Blood (in)	Liberec, CZ, 1998
LUH 5834	NIPH 1118 [†]	Nemeč <i>et al.</i> (2000)	Wound (out)	Sedlčany, CZ, 1999
LUH 5835	NIPH 1120 [†]	Nemeč <i>et al.</i> (2000)	Urine (out)	Příbram, CZ, 1999
LUH 4761	72a [†]	Tjernberg & Ursing (1989)	Urine (out)	Malmö, SE, 1980
LUH 4762	93 [†]	Tjernberg & Ursing (1989)	Blood (in)	Malmö, SE, 1980
LUH 4763	119 [†]	Tjernberg & Ursing (1989)	Urine (out)	Malmö, SE, 1980
LUH 4766	166 [†]	Tjernberg & Ursing (1989)	Wound (in)	Malmö, SE, 1981
LUH 4768	175 [†]	Tjernberg & Ursing (1989)	Wound (in)	Malmö, SE, 1981
RUH 1501			Hairy skin (in)	Rotterdam, NL, 1985
RUH 3329	Patient K [†]	Horrevorts <i>et al.</i> (1995)	Blood (in)	Nijmegen, NL, 1990
LUH 3292 [†]		Bernards <i>et al.</i> (1997)	Blood (in)	Leiden, NL, 1995
LUH 3299 [†]		Bernards <i>et al.</i> (1997)	Blood (in)	Leiden, NL, 1995
LUH 3140		A. T. Bernards	Toes (in)	Enschede, NL, 1995
LUH 3059		A. T. Bernards	Blood (in)	Enschede, NL, 1995
LUH 3324	610/1994 [‡]	J. G. M. Koelman	Blood	Amsterdam, NL, 1994
LUH 4739	84 [†]	Bouvet & Grimont (1986)	Blood	Unknown
LUH 4828	1614/96 [‡]	D. A. Caugant	Abscess (out)	Kristiansand, NO, 1996
Phenon 2 (<i>A. schindleri</i> sp. nov.)				
LUH 5832 [†]	NIPH 1034 [†] = LMG 19576 [†] = CNCTC 6736 [†]	Nemeč <i>et al.</i> (2000)	Urine (out)	Příbram, CZ, 1998
LUH 4590	NIPH 228 [†]	Nemeč <i>et al.</i> (2000)	Vagina (out)	Praha, CZ, 1994
LUH 4591	NIPH 257 [†]	Nemeč <i>et al.</i> (2000)	Urine (out)	Hluboká nad Vltavou, CZ, 1993
LUH 4594	NIPH 285 [†]	Nemeč <i>et al.</i> (2000)	Throat (out)	Příbram, CZ, 1994
LUH 4595	NIPH 286 [†]	Nemeč <i>et al.</i> (2000)	Ear (out)	Příbram, CZ, 1994
LUH 4597	NIPH 291 [†]	Nemeč <i>et al.</i> (2000)	Nasal swab (out)	Příbram, CZ, 1994
LUH 4598	NIPH 293 [†]	Nemeč <i>et al.</i> (2000)	Cervix (out)	Sedlčany, CZ, 1994
LUH 4599	NIPH 296 [†]	Nemeč <i>et al.</i> (2000)	Cervix (out)	Sedlčany, CZ, 1994
LUH 4612	NIPH 369 [†]	Nemeč <i>et al.</i> (2000)	Cervix (out)	Sedlčany, CZ, 1994
LUH 4615	NIPH 383 [†]	Nemeč <i>et al.</i> (2000)	Nasal swab (out)	Milín, CZ, 1996
LUH 5825	NIPH 883 [†]	Nemeč <i>et al.</i> (2000)	Urine (out)	Příbram, CZ, 1998
LUH 5826	NIPH 900 [†]	Nemeč <i>et al.</i> (2000)	Conjunctiva (out)	Sedlčany, CZ, 1998
LUH 5827	NIPH 904 [†]	Nemeč <i>et al.</i> (2000)	Urine (out)	Příbram, CZ, 1998
LUH 5939	NIPH 907 [†]	Nemeč <i>et al.</i> (2000)	Nasal swab (out)	Příbram, CZ, 1998
LUH 5828	NIPH 933 [†]	Nemeč <i>et al.</i> (2000)	Vagina (out)	Příbram, CZ, 1998
LUH 4760	60 [†]	Tjernberg & Ursing (1989)	Urine (in)	Malmö, SE, 1980
LUH 4764	120 [†]	Tjernberg & Ursing (1989)	Pleural effusion (in)	Malmö, SE, 1980
LUH 4765	129 [†]	Tjernberg & Ursing (1989)	Urine	Malmö, SE, 1980
RUH 203 [†]		Dijkshoorn <i>et al.</i> (1998)	Liquor (out)	Rotterdam, NL, 1983
LUH 4742	594 [‡]	P. J. M. Bouvet	Skin	Unknown
LUH 4743	585 [‡]	P. J. M. Bouvet	Skin	Unknown
LUH 4744	586 [‡]	P. J. M. Bouvet	Skin	Unknown

* If known, specimens from outpatients (out) or inpatients (in) are indicated.

[†] Strain designation used in a previous publication.

[‡] Strain designation as received.

status of these groups by a polyphasic analysis. For this purpose, the collection of Czech strains was enlarged with strains from other European countries that showed characters similar to those of the two phenons.

METHODS

Strains. The 29 strains of phenon 1 and 22 strains of phenon 2 investigated in this study are listed in Table 1. The Czech strains ($n = 30$) were those from the previous study (Nemeč *et al.*, 2000). Additionally, 21 strains were selected from a set

Table 2. Biochemical characteristics of phenon 1 (*A. ursingii* sp. nov.) and phenon 2 (*A. schindleri* sp. nov.)

Data are from this study and from Nemeč *et al.* (2000). Growth on carbon sources was evaluated after 2 and 6 d of incubation. +, Positive for all strains; –, negative for all strains; numbers are percentages of strains giving a positive reaction. All strains utilized DL-lactate and acetate. None of the strains grew at 44 °C, hydrolysed gelatin, produced haemolysis on sheep-blood agar, acidified Hugh & Leifson's medium with D-glucose or utilized DL-4-aminobutyrate, β -alanine, L-histidine, malonate, histamine, L-phenylalanine, phenylacetate, laevulinate, citraconate or L-leucine.

Characteristic	Phenon 1 (<i>A. ursingii</i> sp. nov.) (n = 29)	Phenon 2 (<i>A. schindleri</i> sp. nov.) (n = 22)
Growth at 41 °C	–	+*
Growth at 37 °C	+*	+
Utilization of:		
Citrate (Simmons)	+	59
Glutarate	97	95
L-Aspartate	97*	–
Azelate	+	64
D-Malate	+*	95*
4-Hydroxybenzoate	97	64
L-Tartrate	–	18
2,3-Butanediol	–	32
Ethanol	+	95

* Weak growth of some strains.

of about 100 *Acinetobacter* strains isolated by different laboratories that could not be identified as any of the described genomic species. The 21 strains were selected from this set on the basis of phenotypic properties and ARDRA patterns similar to those of the phenon 1 or phenon 2 strains (Nemeč *et al.*, 2000). All 51 strains had the properties of the genus *Acinetobacter* (Juni, 1984); i.e. they were Gram-negative, strictly aerobic, oxidase-negative, non-motile coccobacilli and positive in the transformation assay of Juni (1972).

Phenotypic characterization. The tests described by Nemeč *et al.* (2000) were used, with the following modifications. Carbon-source utilization tests were supplemented with those for laevulinate, citraconate, 4-hydroxybenzoate, L-tartrate, L-leucine, 2,3-butanediol, ethanol and acetate. The test for *trans*-aconitate utilization was omitted since it may give irreproducible results with some phenon 1 strains. Production of pigments was tested on glycerol-containing media A and B as described by King *et al.* (1954). All tests were performed at 30 °C unless indicated otherwise.

ARDRA. Amplified 16S rDNA was obtained by PCR and analysed by restriction digestion with six restriction endonucleases (*Cfo*I, *Alu*I, *Mbo*I, *Rsa*I, *Msp*I and *Bfa*I) as described previously (Nemeč *et al.*, 2000). Interpretation of ARDRA patterns was based on the positions of the fragments of molecular size ≥ 100 bp. The patterns were numbered according to the scheme of Dijkshoorn *et al.* (1998), supplemented by Seifert *et al.* (1997) and Nemeč *et al.* (2000).

AFLP fingerprinting. AFLP was performed according to Koeleman *et al.* (1998), with some modifications. DNA was purified as described by Boom *et al.* (1990) and adapters were as described by Vos *et al.* (1995). Restriction and ligation were performed simultaneously at 37 °C for 3 h in a 10 μ l volume with 10–50 ng template DNA, 1 U *Eco*RI (Amersham Pharmacia Biotech), 1 U *Mse*I (New England BioLabs), 4 U T4 DNA ligase (Amersham Pharmacia

Biotech), 1 \times T4 DNA ligase buffer, 500 ng BSA, 50 mM NaCl, 2 pmol *Eco*RI adapters and 20 pmol *Mse*I adapters. After incubation, the mixture was diluted with 10 mM Tris/HCl, 0.1 mM EDTA (pH 8.0) to a final volume of 200 μ l. Five microlitres diluted mixture was added to a final volume of 10 μ l reaction mixture containing 20 ng Cy5-labelled *Eco*RI+A primer (Cy5-GACTGCGTACCAATTCa-3'; where a is a selective A base), 60 ng *Mse*I+C primer (5'-GATGAGTCCTGAGTAAc-3'; where c is a selective C base), 1 \times *Taq* polymerase buffer, 1.5 mM MgCl₂, 0.2 mM (each) dNTP and 1 U Goldstar *Taq* DNA polymerase (Eurogentec). Amplification with a Progene thermocycler (Techne) was as follows: 2 min at 72 °C and 2 min at 94 °C; one cycle of 30 s at 94 °C, 30 s at 65 °C and 60 s at 72 °C; 12 cycles of 30 s at 94 °C, 30 s at a temperature of 0.7 °C lower than the previous cycle, starting at 64.3 °C, followed by 60 s at 72 °C; 23 cycles of 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C; and a final cycle of 10 min at 72 °C. PCR products were mixed with 3 μ l formamide containing 0.5% dextran blue, heated for 5 min at 95 °C and cooled on ice. Samples of 3 μ l were loaded on a denaturing polyacrylamide gel (ReproGel High Resolution; Amersham Pharmacia Biotech) with 200 mm standard thermoplates. Fragment separation was performed using the ALFexpress II DNA analysis system (Amersham Pharmacia Biotech) for 500 min at 55 °C and 30 W constant power with 2 s sampling intervals. The peak patterns generated were converted to TIF files, which were analysed by the BIO NUMERICS 2.0 software package (Applied Maths). Fragments in the range 50–500 bp were used for cluster analysis. Pearson's product-moment coefficient (*r*) was used as a measure of similarity and grouping was obtained by the unweighted pair group average linked method (UPGMA).

DNA–DNA hybridization. The two-step elution procedure was used to determine DNA–DNA relatedness (Tjernberg *et al.*, 1989). By this method, ¹²⁵I-labelled DNA probes from strains LUH 3792^T (phenon 1) and LUH 5832^T (phenon 2) were hybridized on a filter with unlabelled DNAs of the

Table 3. ARDRA patterns of phenon 1 and phenon 2 strains

Data were from this study and from Nemeč *et al.* (2000). Pattern designation according to Dijkshoorn *et al.* (1998) and Nemeč *et al.* (2000). ND, Not determined; New, novel patterns.

Strain(s)	Restriction pattern with:					
	<i>CfoI</i>	<i>AluI</i>	<i>MboI</i>	<i>RsaI</i>	<i>MspI</i>	<i>BfaI</i>
Phenon 1 (<i>A. ursingii</i> sp. nov.)						
LUH 3792 ^T , LUH 3292, LUH 3299, LUH 4592, LUH 4614, LUH 4622, LUH 4762, LUH 4763, LUH 4766, LUH 4828, LUH 5829, LUH 5830, LUH 5834, RUH 1501, RUH 3329	1	4	3	5	3	ND
LUH 3059, LUH 3793, LUH 4582, LUH 4768	1	4+nw*	3	4	3	ND
LUH 3140, LUH 5767, LUH 5835	1	4+nw*	3	4+5	3	ND
LUH 3324, LUH 4761	1	4+nw*	1+3	4+5	3	ND
LUH 4739	1	4	1+3	5	3	ND
LUH 4618	1	4	3	4+5	3	ND
LUH 5833	1	4	1+3	4+5	3	ND
LUH 5831	1	4	3	2+5	3	ND
LUH 4613	1	4†	1+3	2+5	3	ND
Phenon 2 (<i>A. schindleri</i> sp. nov.)						
LUH 5832 ^T , LUH 4591, LUH 4595, LUH 4597, LUH 4598, LUH 4599, LUH 4615, LUH 4742, LUH 4760, LUH 4764, LUH 5825, LUH 5827, LUH 5828, LUH 5939, RUH 203	1+5	2+4‡	1	2	2	10
LUH 4590, LUH 4594, LUH 4612, LUH 4743, LUH 4744, LUH 5826	1+5	2	1	2	2	10
LUH 4765	5	New	1	2	2	New

* A combined *AluI* pattern, tentatively interpreted as the mixture of pattern 4 and a new pattern (Nemeč *et al.*, 2000; Fig. 1).

† Pattern 4 containing an additional, weak band of approximately 223 bp; this pattern is highly similar to combined *AluI* pattern 2+4 (Nemeč *et al.*, 2000).

‡ The band (220 bp) specific for *AluI* pattern 2 was diffuse in all strains (Nemeč *et al.*, 2000; Fig. 1).

phenon 1 and phenon 2 strains and reference strains of all described *Acinetobacter* genomic species. The amount of DNA released from the filter was measured at two temperatures, at 7 °C below the thermal melting midpoint of the homologous duplex and at 100 °C. The amount of DNA released in the first step expressed as a percentage of the total amount of eluted DNA at 100 °C (%DR7) was the criterion for inclusion of strains in a species, with the intraspecies and interspecies values for %DR7 being ≤ 26 and ≥ 37 , respectively (Tjernberg *et al.*, 1989). Each %DR7 value was calculated as a mean of at least two hybridization experiments.

16S rDNA sequencing and comparative analysis. A fragment of the 16S rRNA gene (corresponding to positions 10–1507 in the *Escherichia coli* numbering system) of three phenon 1 strains (LUH 3792^T, LUH 3299, LUH 4763) and three phenon 2 strains (LUH 5832^T, LUH 4591, LUH 4760) was sequenced as described by Vaneechoutte *et al.* (2000). The 16S rDNA sequences obtained for phenon 1 and phenon 2 strains were compared with the sequences representing all described *Acinetobacter* genomic species, i.e. 21 sequences determined by Ibrahim *et al.* (1997) (EMBL accession numbers Z93434–Z93454) and the sequence of '*A.*

venetianus' strain RAG-1 (AJ295007), and the sequences of *Moraxella lacunata* ATCC 17967^T (AF005160) and *Psychrobacter immobilis* ATCC 43116^T (U39399). All steps of the comparative sequence analysis were performed by using the GENEBASE software package (Applied Maths). Firstly, pairwise alignment using UPGMA was carried out with a gap penalty of 100%, a unit gap cost of 20% and an ambiguity cost of 50% of the mismatch cost. Subsequently, global alignment with *P. immobilis* as the outgroup was carried out on the region corresponding to positions 67–1444 of the 16S rRNA gene of *E. coli*, with costs as above. Finally, a similarity matrix of the aligned sequences was constructed by global alignment homology calculation and a gap penalty of 20%. The neighbour-joining method was used to construct the dendrogram based on this similarity matrix.

RESULTS AND DISCUSSION

Phenotypic characteristics

Colonies of all strains grown on nutrient agar after 24 h were circular, convex, smooth and slightly opaque with entire margins. The colonies of phenon 1 strains

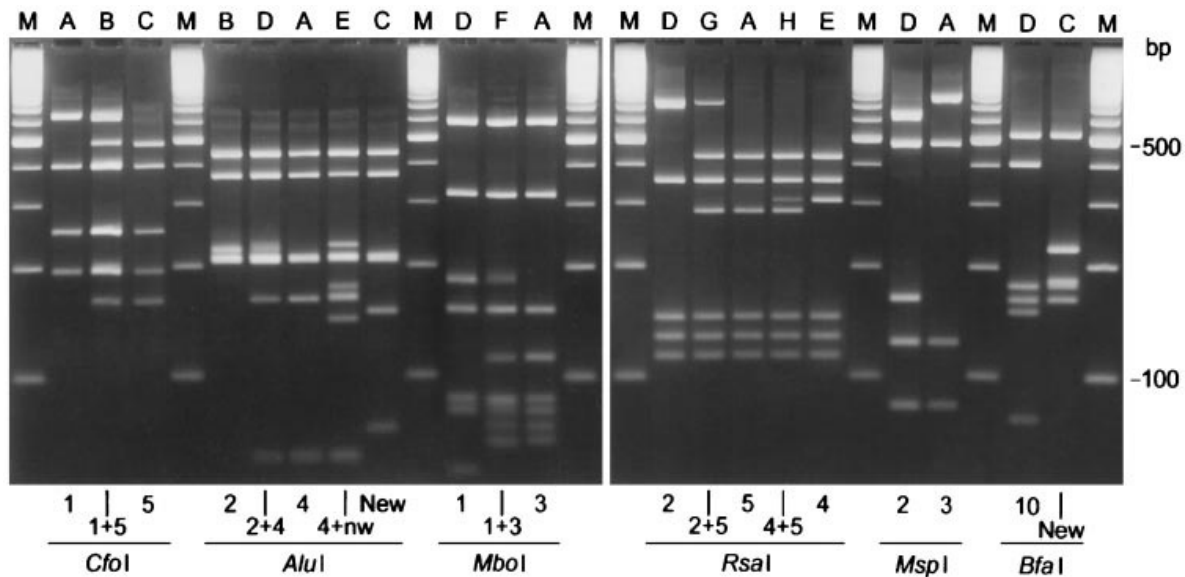


Fig. 1. Overview of the ARDRA patterns found in phenon 1 (*A. ursingii* sp. nov.) and phenon 2 (*A. schindleri* sp. nov.) strains. Strains are indicated by upper-case letters above the lanes: A, LUH 3792^T (phenon 1); B, LUH 4594 (phenon 2); C, LUH 4765 (phenon 2); D, LUH 5832^T (phenon 2); E, LUH 3793 (phenon 1); F, LUH 4761 (phenon 1); G, LUH 4613 (phenon 1); H, LUH 4618 (phenon 1). Lanes M, molecular size markers (100-bp ladder). Pattern designations for the various enzymes are given below the lanes.

were respectively 1–1.5 mm and 1.5–3 mm in diameter after 24 h and 48 h of incubation. The colonies of phenon 2 strains were respectively 1.5–2.5 and 2–4.5 mm in diameter after 24 h and 48 h of incubation. Some phenon 2 strains (e.g. LUH 5832^T, LUH 4615 and LUH 4764) produced diffuse, light yellowish-brown pigment on King's medium A and were surrounded by dark greenish zones on sheep-blood agar.

Biochemical test results are given in Table 2. The strains of phenon 1 were, with few exceptions, biochemically uniform, while those of phenon 2 varied in the utilization of citrate (Simmons), azelate, 4-hydroxybenzoate, L-tartrate and 2,3-butanediol. Growth of some strains of both phenons on D-malate and of some phenon 1 strains on L-aspartate was weak after 6 d and became more apparent after prolonged incubation (up to 10 d).

ARDRA

ARDRA patterns of the phenon 1 and phenon 2 strains are summarized in Table 3 and Fig. 1. Most phenon 1 strains shared the recently described *RsaI* pattern 5 (Nemec *et al.*, 2000), which differs slightly from pattern 4 in migration of a fragment of about 300 bp (Fig. 1). Based on the analysis of the 16S rDNA sequences in the present study, this difference can be explained by the presence of an additional *RsaI* restriction site responsible for a 22 bp truncation of the fragment in *RsaI* pattern 5. Accordingly, the previously published *RsaI* patterns 4 of strains LUH 3292, LUH 3299 and LUH 3329 (Bernards *et al.*, 1997;

Dijkshoorn *et al.*, 1998) were reinterpreted as *RsaI* patterns 5. Some of the ARDRA patterns appeared to be mixtures of two known single patterns. Of these, *AluI* 2+4 and *CfoI* 1+5 patterns were found in most phenon 2 strains. However, the band specific for *AluI* pattern 4 (162 kb) was very weak in some of these strains (e.g. RUH 203 and LUH 4760) and could only be seen clearly when the gel was overloaded with DNA. Similarly, RUH 203 and LUH 4590 yielded very faint bands of 160 and 479 kb specific for *CfoI* pattern 5. This observation may explain the difference between the published *CfoI* pattern 1 and *AluI* pattern 2 of strain RUH 203 (Dijkshoorn *et al.*, 1998) and those of the present study.

AFLP fingerprinting

Reproducibility of AFLP as determined by testing several control strains was always higher than 90% (data not shown). Cluster analysis of the phenon 1 and 2 strains was performed together with a total of 200 strains from all described *Acinetobacter* genomic species (identified by DNA–DNA hybridization). The strains of each of the described genomic species formed a separate cluster at a cut-off level of about 50% (data not shown). Clustering of all phenon 1 and phenon 2 strains and one representative strain of each described *Acinetobacter* genomic species is shown in Fig. 2. The strains of phenon 1 and phenon 2 grouped in two clusters at levels of 67 and 63% and were clearly separated from each other, and from all other strains at 33 and 20%, respectively.

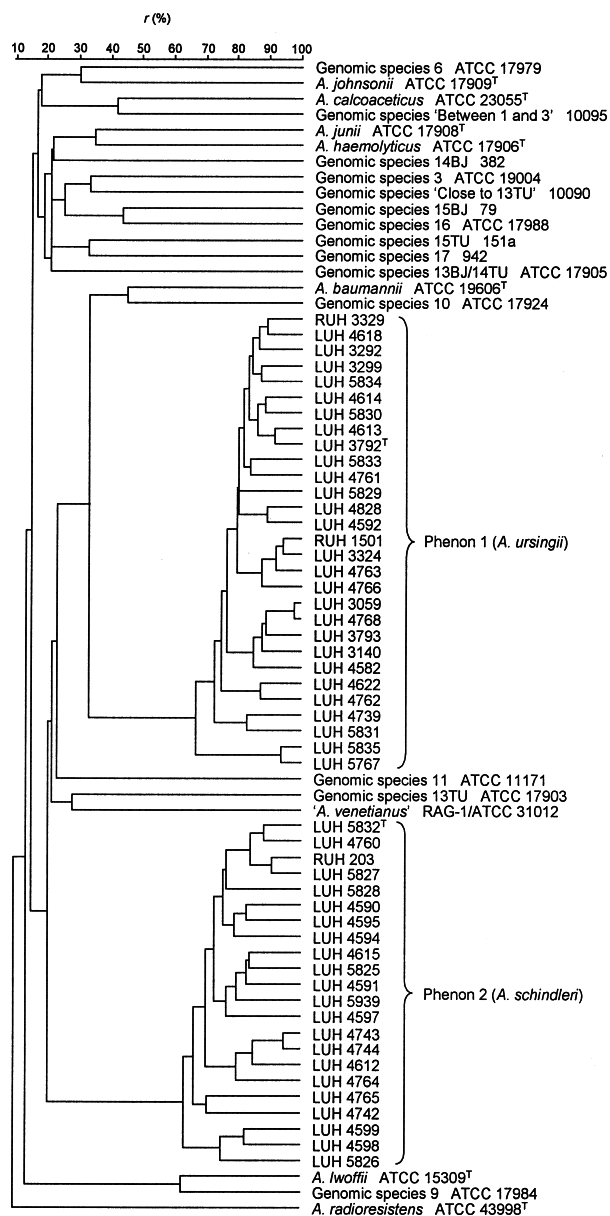


Fig. 2. UPGMA/product-moment cluster analysis of the AFLP fingerprints of 29 strains of phenon 1 (*A. ursingii* sp. nov.), 22 strains of phenon 2 (*A. schindleri* sp. nov.) and 22 strains representing all hitherto-described (genomic) species of the genus *Acinetobacter*. The latter strains are designated by either the ATCC numbers or the numbers used in previous DNA–DNA hybridization studies (Tjernberg & Ursing, 1989; Bouvet & Jeanjean, 1989; Gerner-Smidt & Tjernberg, 1993). Levels of correlation are expressed as percentages of similarity for convenience.

DNA–DNA hybridization

The %DR7 values obtained with radiolabelled DNA from strains LUH 3792^T and LUH 5832^T are summarized in Table 4. The intraphenon range of the %DR7 values corresponded to the intraspecies variability of %DR7 values found previously (Tjernberg *et al.*, 1989), the only exception being LUH 4590 (phenon 2), with %DR7 = 30. However, although the latter

value was relatively high, it was significantly lower than the values found for hybridization with both the reference strains of the described genomic species and phenon 1 strains. Thus, the %DR7 values support the conclusion that the strains of phenon 1 and phenon 2 represent novel, distinctive genomic groups.

16S rDNA sequence analysis

The 16S rDNA sequences of phenon 1 strains LUH 3792^T, LUH 3299 and LUH 4763 were identical, and the sequences of phenon 2 strains LUH 5832^T, LUH 4591 and LUH 4760 were nearly identical (99.4% similarity). A dendrogram based on the comparison of these sequences with those representing known *Acinetobacter* genomic species and the closest genera is shown in Fig. 3. Both phenon 1 and phenon 2 strains clustered with the other members of the genus *Acinetobacter* and were well separated from their neighbours. The similarity values between the 16S rDNA sequence of the phenon 1 strains and those of the other members of the genus *Acinetobacter* were in the range 95.4–97.3%; the similarity between the sequences of the phenon 2 strains and those of the other members of the genus ranged from 95.4 to 98.0%. The lowest intra-generic 16S rDNA sequence similarity (95.4%) was observed between phenon 1 and phenon 2 strain LUH 4760.

Taxonomic status of phenon 1 and phenon 2

The results of DNA–DNA hybridization and AFLP confirmed that phenon 1 and phenon 2 represent two distinctive genomic species, different from all hitherto-described *Acinetobacter* genomic species. Furthermore, comparative analysis of 16S rDNA sequences indicated that phenon 1 and phenon 2 formed two distinct lineages within the genus *Acinetobacter*. Both phenons could be differentiated from the other genomic species of the genus and from each other by ARDRA patterns and biochemical characters (see below). On the basis of these findings, phenon 1 and phenon 2 described by Nemeč *et al.* (2000) represent two novel species of the genus *Acinetobacter*, for which the respective names *Acinetobacter ursingii* sp. nov. and *Acinetobacter schindleri* sp. nov. are proposed.

Differentiation and identification

The array of 19 biochemical tests suggested by Bouvet & Grimont (1987) allowed unambiguous identification of almost all strains of *A. ursingii* and *A. schindleri*. Comparison of our results with those of previous studies (Bouvet & Grimont, 1987; Gerner-Smidt *et al.*, 1991; Vanechoutte *et al.*, 1999) showed that both novel species could be differentiated from most other genomic species of the genus *Acinetobacter* by their inability to grow at 44 °C, to oxidize D-glucose, to hydrolyse gelatin and to utilize DL-4-aminobutyrate, β-alanine, L-histidine, malonate, histamine, L-phenylalanine and phenylacetate. Growth at 41 and 37 °C

Table 4. Results of DNA–DNA hybridization using the %DR7 coefficient

The reference strains were those used in the studies of Tjernberg & Ursing (1989) and Bouvet & Jeanjean (1989), strains 10095 and 10090 (Gerner-Smidt & Tjernberg, 1993) and '*A. venetianus*' strains C3 and RAG-1 (Di Cello *et al.*, 1997; Vaneechoutte *et al.*, 1999). Values are means \pm SD, with the range in parentheses.

Source of unlabelled DNA	Labelled DNA from LUH 3792 ^T (phenon 1, <i>A. ursingii</i> sp. nov.)	Labelled DNA from LUH 5832 ^T (phenon 2, <i>A. schindleri</i> sp. nov.)
Phenon 1 strains (<i>n</i> = 29)	12 \pm 4 (7–25)	–
Reference strains of the known genomic species (<i>n</i> = 22) and phenon 2 strains LUH 4590 and LUH 4765	46 \pm 5 (37–54)	–
Phenon 2 strains (<i>n</i> = 22)	–	22 \pm 4 (14–30)
Reference strains of the known genomic species (<i>n</i> = 22) and phenon 1 strains LUH 3792 ^T , LUH 4761 and LUH 4828	–	57 \pm 4 (49–70)

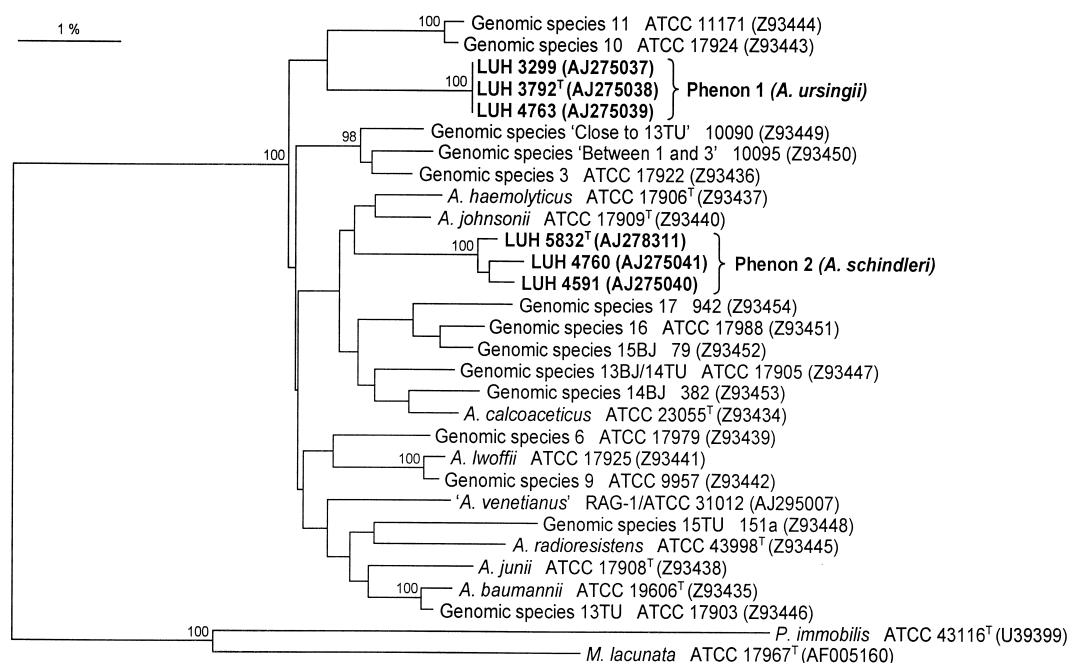


Fig. 3. Rooted 16S rDNA sequence-based tree showing the relationship of phenon 1 (*A. ursingii* sp. nov.), phenon 2 (*A. schindleri* sp. nov.), the other members of the genus *Acinetobacter*, *Moraxella lacunata* and *Psychrobacter immobilis* (the outgroup). The tree was constructed using the neighbour-joining method. The numbers at the branching points are the proportions of 100 bootstrap resamplings that support the tree topology (only values above 90% are shown). EMBL accession numbers are given in parentheses. Bar, 1% estimated sequence divergence.

and utilization of glutarate and L-aspartate were the most useful tests for differentiating *A. ursingii* and *A. schindleri* from each other and from *A. junii*, *A. johnsonii*, *A. lwoffii* and genomic species 15TU (Table 5). Only two strains could not be identified unambiguously; *A. ursingii* LUH 4614 failed to grow on L-aspartate and therefore could not be differentiated from *A. schindleri*, while *A. schindleri* LUH 5939 did not utilize glutarate and consequently could not be distinguished from genomic species 15TU and *A. lwoffii*. However, LUH 5939 could be differentiated from *A. lwoffii* by its ability to utilize 4-hydroxy-

benzoate, which is not included in the identification scheme of Bouvet & Grimont (1987).

None of the ARDRA profiles of the *A. ursingii* and *A. schindleri* strains have been observed previously in any of the known genomic species (Dijkshoorn *et al.*, 1998; Seifert *et al.*, 1997; Vaneechoutte *et al.*, 1999). All but one of the *A. schindleri* strains yielded *Bfa*I pattern 10, which may be particularly useful in their differentiation from *A. johnsonii* strains that have highly similar pattern combinations with the other enzymes used in ARDRA (Seifert *et al.*, 1997). A total of nine different

Table 5. Phenotypic characters useful for discrimination of *A. ursingii* and *A. schindleri* and for their differentiation from phenotypically similar (genomic) species

Data for *A. junii*, *A. johnsonii*, *A. lwoffii* and genomic species 15TU were taken from Gerner-Smidt *et al.* (1991). +, Positive for 90–100% of strains; –, positive for 0–10% of strains; D, positive for 11–89% of strains.

Characteristic	<i>A. ursingii</i>	<i>A. schindleri</i>	<i>A. junii</i>	<i>A. johnsonii</i>	<i>A. lwoffii</i>	Genomic species 15TU
Growth at 41 °C	–	+	D	–	–	D
Growth at 37 °C	+	+	+	–	D	+
Utilization of:						
Glutarate	+	+	–	–	–	–
L-Aspartate	+	–	–	D	–	–

ARDRA profiles were encountered among the *A. ursingii* strains. In spite of this variability, several pattern combinations may be useful for the identification of *A. ursingii*, e.g. the combination of *CfoI* 1, *MboI* 3 or *MboI* 1+3 and *MspI* 3 or the combination of *RsaI* 4 or *RsaI* 5 or *RsaI* 4+5 or *RsaI* 2+5 and *MspI* 3.

Clinical importance

The available clinical and epidemiological data suggest that *A. ursingii* and *A. schindleri* differ in their distribution in patients. While the majority of the *A. schindleri* strains were isolated from non-sterile body sites of outpatients, *A. ursingii* comprised mainly clinically significant isolates from seriously ill hospitalized patients. Almost half of the *A. ursingii* strains were isolated from blood cultures and at least some of them were recovered from patients with diagnosed bacteraemia or septicaemia (Bernards *et al.*, 1997; Horrevorts *et al.*, 1995; Nemeč *et al.*, 2000). Moreover, the identity of typing characters that was found in two epidemiologically related isolates (Nemeč *et al.*, 2000) indicates that *A. ursingii* strains have the potential to spread among patients.

Description of *Acinetobacter ursingii* sp. nov.

Acinetobacter ursingii (ur.sin'gi.i. N.L. gen. masc. n. *ursingii* in honour of Jan Ursing, the recently deceased Swedish bacteriologist and taxonomist).

Characteristics correspond to those of the genus (Juni, 1984). Colonies on nutrient agar after 24 h incubation at 30 °C are approximately 1.0–1.5 mm in diameter, circular, convex, smooth and slightly opaque with entire margins. Growth occurs at 37 °C but not at 41 °C. Acid is not produced from D-glucose, sheep blood is not haemolysed and gelatin is not hydrolysed. DL-Lactate, citrate (Simmons), azelate, D-malate, ethanol and acetate are utilized as sole sources of carbon and energy. Glutarate, L-aspartate and 4-hydroxybenzoate are utilized by most strains. DL-4-Aminobutyrate, β -alanine, L-histidine, malonate, histamine, L-phenylalanine, phenylacetate, laevulinate, citraconate, L-tartrate, L-leucine and 2,3-butanediol are not utilized.

The type strain is LUH 3792^T (= NIPH 137^T = LMG 19575^T = CNCTC 6735^T), isolated from blood of a hospitalized patient with endocarditis. This strain utilizes glutarate, L-aspartate and 4-hydroxybenzoate. The restriction patterns of amplified 16S rDNA of the type strain are *CfoI* 1, *AluI* 4, *MboI* 3, *RsaI* 5, *MspI* 3. The EMBL accession number for its 16S rDNA sequence is AJ275038.

Description of *Acinetobacter schindleri* sp. nov.

Acinetobacter schindleri (schin'dle.ri. N.L. gen. masc. n. *schindleri* in honour of Jiří Schindler, Czech microbiologist and taxonomist).

Characteristics correspond to those of the genus (Juni, 1984). Colonies on nutrient agar after 24 h incubation at 30 °C are approximately 1.5–2.5 mm in diameter, circular, convex, smooth and slightly opaque with entire margins. Growth occurs at 41 °C but not at 44 °C. Acid is not produced from D-glucose, sheep blood is not haemolysed and gelatin is not hydrolysed. DL-Lactate and acetate are utilized as sole sources of carbon and energy. Glutarate, D-malate and ethanol are utilized by most strains. Various numbers of strains utilize citrate (Simmons), azelate, 4-hydroxybenzoate, L-tartrate and 2,3-butanediol. DL-4-Aminobutyrate, L-aspartate, β -alanine, L-histidine, malonate, histamine, L-phenylalanine, phenylacetate, laevulinate, citraconate and L-leucine are not utilized.

The type strain is LUH 5832^T (= NIPH 1034^T = LMG 19576^T = CNCTC 6736^T), isolated from urine of a male outpatient with cystitis. This strain utilizes citrate (Simmons), glutarate, D-malate, 4-hydroxybenzoate and ethanol but not azelate, L-tartrate or 2,4-butanediol. The restriction patterns of the amplified 16S rDNA of the type strain are *CfoI* 1+5, *AluI* 2+4, *MboI* 1, *RsaI* 2, *MspI* 2, *BfaI* 10. The EMBL accession number for its 16S rDNA sequence is AJ278311.

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Isolation of *Moraxella canis* from an Ulcerated Metastatic Lymph Node

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***Moraxella canis* was isolated in large numbers from an ulcerated supraclavicular lymph node of a terminal patient, who died a few days later. Although the patient presented with septic symptoms and with a heavy growth of gram-negative diplococci in the lymph node, blood cultures remained negative. *M. canis* is an upper-airway commensal from dogs and cats and is considered nonpathogenic for humans, although this is the third reported human isolate of this species.**

Case report. A 51-year-old patient who was a 55-pack-per-year smoker and who had a long history of serious, chronic obstructive pulmonary disease was sent to the hospital in October 1998 because of dyspnea. A primary bronchial carcinoma was diagnosed. Biopsy of the left supraclavicular lymph node revealed a moderately differentiated adenocarcinoma. Palliative radiotherapy was started but was stopped in December 1998, and the patient was sent home during the first week of January.

The patient was in a cachectic, immunodepressed condition, presented with fever and chills, and had a large ulcer at the left supraclavicular lymph node. A differential diagnosis of sepsis and tumor was made. Computed tomography of the thorax showed a large necrotic nodus at the left supraclavicular lymph node and a tumor at the right lower lobe in association with pleural fluid effusion and a diffuse metastasized bronchial carcinoma. *Staphylococcus aureus* in small numbers and *Moraxella canis* in large numbers were isolated from the ulcer wound. Blood cultures remained negative. The patient was discharged 2 days later and died shortly thereafter at home.

The oxidase-positive gram-negative diplococci were given a preliminary identification as *M. canis* after observation of brown pigmentation of the Mueller-Hinton II agar (MHAI; BBL Becton Dickinson, Cockeysville, Md.), used routinely for disk diffusion susceptibility testing. This characteristic was present in 15 of the 16 previously described isolates of *M. canis*, while absent in all other *Moraxella* species (2). Further differentiation from other *Moraxella* species was possible on the basis of a positive DNase reaction, acetate assimilation positivity, and a positive gamma glutamyl aminopeptidase reaction (2).

The isolate (LBV436) was resistant to ampicillin and susceptible to co-trimoxazole, doxycycline, fucidic acid, vancomycin, rifampin, gentamicin, and quinolones. Production of β -lactamase has been demonstrated in some strains of *M. canis* (2), and this strain was also β -lactamase positive.

M. canis, together with *Moraxella catarrhalis*, *Moraxella cuniculi*, *Moraxella caviae*, *Moraxella ovis*, and *Moraxella* sp. incertae sedis strain NCTC 4103 belong to the coccal moraxellae, which, in contrast to the bacillary moraxellae, all exhibit DNase

activity. *M. canis* and strain NCTC 4103 differ from all other coccoid moraxellae by the production of gamma glutamyl aminopeptidase and the ability to grow on MHAI at room temperature. Moreover, *M. canis* and, to a weaker extent, strain NCTC 4103 produce a brownish pigment on this growth medium, which is a feature not shown by any other moraxellae.

To our knowledge, this is the third isolation of *M. canis* from humans. The first isolate (N7^T, CCUG 8415A^T) was from a dog bite wound in a Swedish female in 1979, for which no further clinical data are available (2). Wüst et al. (7) described a blood culture isolate (U33, BLU8387) obtained in 1988 from a 61-year-old alcoholic Swiss male with bleeding esophageal varices who was hospitalized for symptoms suggesting pneumonia. The cachectic immunodepressed condition of our patient is comparable to that of the Swiss patient (7). All other known isolates of this species are commensals isolated from dog saliva (P37, Paris) (2) or swabs from dog muzzles (one was from a cat muzzle) (2), from which *M. canis* could be cultured only after suppression of other commensal bacteria with a selective medium (4).

Sequence determination of the first 450 to 700 bp of the PCR-amplified 16S rRNA gene was carried out for isolate LBV436, for four of the previously collected *M. canis* strains (O18, P37, U33, and W4), for strain NCTC 4103, for an unidentified gram-negative diplococcus isolated from a dog bite (MOR32), and for a *Moraxella* strain producing yellowish pigment on MHAI (LBV438). The complete sequence was determined for the *M. canis* type strain N7. Amplification was done by PCR with the primers 5'-AGT TTG ATC CTG GCT CAG and 5'-TAC CTT GTT ACG ACT TCG TCC CA. The reactions were performed in a final reaction mixture of 50 μ l containing 25 μ l of Master Mix (Qiagen, Hilden, Germany), a 0.2- μ M concentration of each primer, and 5 μ l of a DNA suspension obtained by alkaline lysis. Alkaline lysis was done by suspending one colony in 20 μ l of 0.25% sodium dodecyl sulfate–0.05 N NaOH and heating at 95°C for 15 min, followed by a final dilution with 180 μ l of distilled water. The amplification reactions were performed in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, Calif.) with the following cycling parameters: 94°C for 5 min, followed by 3 cycles of 45 s at 94°C, 2 min at 50°C, 1 min at 72°C, and 30 cycles of 20 s at 94°C, 1 min at 50°C, 1 min at 72°C, with a final extension at 72°C for 7 min. The presence of amplification products was checked by electrophoresis on 2% agarose gels stained with ethidium bromide. The amplification products were then pu-

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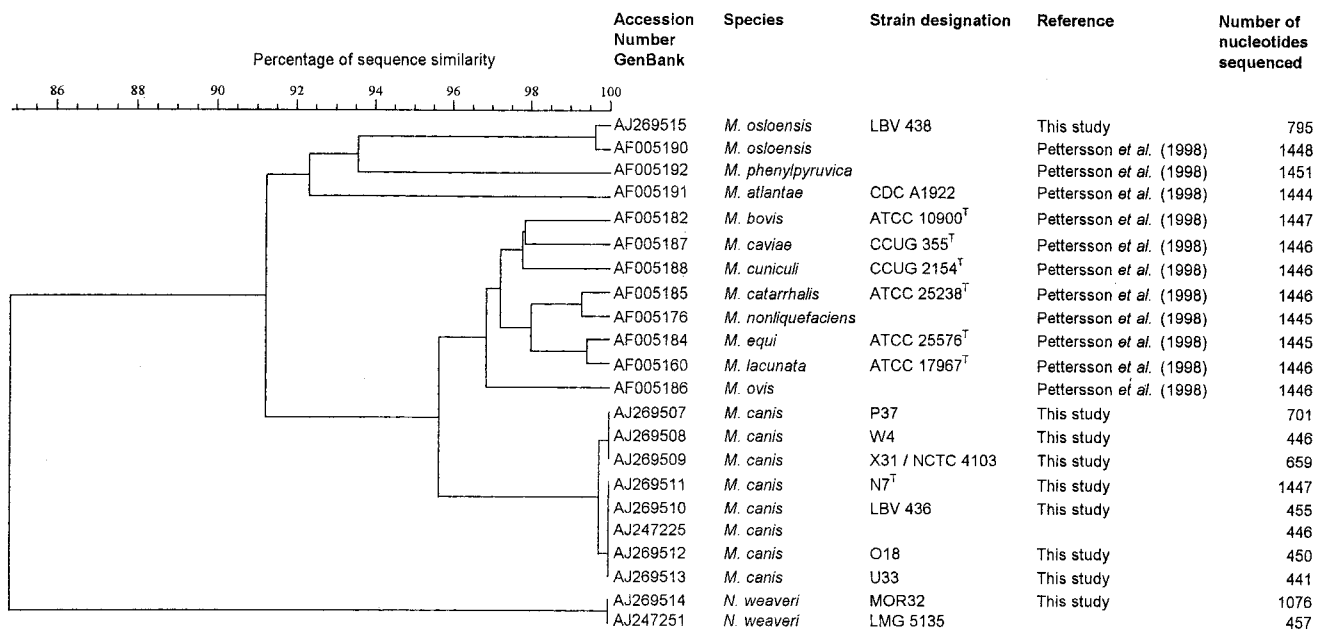


FIG. 1. Dendrogram based on clustering by means of the unweighted pair groups method using arithmetic averages (open gap penalty, 100%; unit gap penalty, 0%) of 16S rDNA sequences published for the genus *Moraxella* and obtained in this study.

rified with the Concert PCR purification kit (Gibco BRL Life Technologies, Merelbeke, Belgium), used according to the manufacturer's instructions. Sequencing was done using the ABI Big Dye cycle sequencing reaction kit with AmpliTaq FS DNA polymerase (Applied Biosystems) with the following primers: 5' AGT TTG ATC CTG GCT CAG (*Escherichia coli* 16S rRNA gene sequence position 8 to 27), 5' CTCCTACGG GAGGCAGCAGT (339 to 358 bp), 5' CAGCAGCCGCGG TAATAC (519 to 536), 5' AACTCAAAGGAATTGACGG (908 to 926), 5' AGTCCCGCAACGAGCGCAAC (1093 to 1112), and 5' GCTACACACGTGCTACAATG (1222 to 1241) (1). Electrophoresis was performed on an ABI 310 analyzer. Analysis of the sequences and clustering was done by Gene-Compar, version 2.0 (Applied Maths, Kortrijk, Belgium).

The clinical isolate reported here revealed 100% 16S rDNA sequence identity with U33 (the clinical isolate described by Wüst *et al.* [7]) and above 99% identity with the other four *M. canis* strains sequenced and with strain NCTC 4103. Less than 96% identity was observed with the 16S rDNA sequences of other *Moraxella* species (3). The dog bite isolate MOR32 was identified as *Neisseria weaveri*, and the *Moraxella* isolate which produced yellow pigmentation on MHAI (LBV438) was identified as *M. osloensis* (Fig. 1).

M. canis can also be differentiated from all other *Moraxella* species by amplification of the tRNA intergenic spacers (6) and determination of the tRNA intergenic-spacer lengths by means of capillary electrophoresis (5). *M. canis* and *M. catarrhalis* had tRNA spacer fragments with lengths of 66, 81, and 215 bp in common, which were absent in *M. caviae*, *M. cuniculi*, *Moraxella lacunata*, *Moraxella nonliquefaciens*, *M. osloensis*, and *M. ovis* and could be differentiated from each other by the presence of a fragment of 193 bp in *M. canis* tDNA PCR fingerprints and the presence of fragments of 167 and 179 bp in *M. catarrhalis* fingerprints.

Despite the fact that *M. canis* was isolated in large numbers from the clinical site, the organism has to be considered an opportunistic pathogen, since this strain was isolated from a heavily debilitated patient, as was the case for the Swiss patient (7). Whether our patient lived in close contact with dogs or cats could not be investigated.

In conclusion, gram-negative oxidase-positive diplococci which produce brownish pigment on MHAI are most probably *M. canis*, a *Moraxella* species that is a commensal of dogs and cats and that exceptionally can be isolated from clinical samples in humans.

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Bacteremia Due to *Moraxella atlantae* in a Cancer Patient

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A gram-negative alkaline phosphatase- and pyrrolidone peptidase-positive rod-shaped bacterium (CCUG 45702) was isolated from two aerobic blood cultures from a female cancer patient. No identification could be reached using phenotypic techniques. Amplification of the tRNA intergenic spacers revealed fragments with lengths of 116, 133, and 270 bp, but no such pattern was present in our reference library. Sequencing of the 16S rRNA gene revealed its identity as *Moraxella atlantae*, a species isolated only rarely and published only once as causing infection. In retrospect, the phenotypic characteristics fit the identification as *M. atlantae* (formerly known as CDC group M-3). Comparative 16S rRNA sequence analysis indicates that *M. atlantae*, *M. lincolni*, and *M. osloensis* might constitute three separate genera within the *Moraxellaceae*. After treatment with amoxicillin-clavulanic acid for 2 days, fever subsided and the patient was dismissed.

CASE REPORT

In April 2001 a 31-year-old female patient was admitted to the oncology department with a history of intermittent fever for more than 1 week and with complaints of right hypochondrial pain and rectal cramps. The patient had a rectal adenocarcinoma that was diagnosed 1 year previously, with recent massive liver metastasis which had been treated with an anterior resection and with palliative chemotherapy (irinotecan and 5-fluorouracil) for 10 months. Clinical examination revealed a relapse of the rectal cancer at the anastomosis and a hepatomegaly of 8 cm. X-ray examination of the thorax revealed no infiltrates. Three pairs of blood cultures and a urine culture were carried out, with the latter being negative. Streptococci of the viridans group were isolated from one aerobically incubated culture bottle. A gram-negative rod, susceptible to all antibiotics tested and later identified as *Moraxella atlantae* (CCUG 45702), was isolated from the two other aerobic bottles. The anaerobic cultures remained negative. The patient was treated with amoxicillin-clavulanic acid (875 mg) two times a day, resulting in the disappearance of the fever after 2 days, whereafter the patient was dismissed. A computed tomography of the abdomen 7 days later showed an abscess around the rectum, which was drained with evacuation of 100 ml of serous fluid. Culture of this fluid remained negative. The patient died 4 months later as a result of her underlying disease.

The blood culture bottles (FA aerobic and SN anaerobic bottles; Organon Teknika, Boxtel, The Netherlands) were inoculated with 10 ml of blood and incubated in the Bact/Alert 3D system (Organon Teknika). Gram staining showed short,

plump, rod-shaped to coccoid gram-negative rods. On tryptic soy agar plus 5% sheep blood (Becton Dickinson, Erembodegem, Belgium), colonies were small and grayish, with twitching motility and corroding activity. The isolate grew well on MacConkey agar, in contrast to most other *Moraxella* sp. It was nonmotile. Catalase and oxidase were positive. No sugars were acidified, either fermentatively or oxidatively. No growth was observed on triple sugar iron agar. Nitrate reduction and production of indol, urease, phenylalanine deaminase, lysine and ornithine decarboxylases, and arginine dihydrolase were negative. Using Rosco Diatabs (Taastrup, Denmark), tributyrin and proline aminopeptidase were negative, but alkaline phosphatase was positive and pyrrolidone peptidase was strongly positive. The code obtained by API 20 NE (BioMérieux, Marcy L'Étoile, France) strips was 0000004, corresponding to *Moraxella* sp. (82.3%). No identification was obtained when the ID 32 GN (BioMérieux) strips were used. This phenotypic profile did not initially lead to an identification, but in retrospect it was found to be consistent with the genotypic identification as *M. atlantae*.

Susceptibility was tested with the disk diffusion technique. No criteria are available for interpretation of the results, but because of the large inhibition zones, the strain was considered to be susceptible to all tested antibiotics, namely ampicillin, cotrimoxazole, cefuroxime, gentamicin, colimycin, temocillin, and ciprofloxacin.

Since no initial phenotypic identification could be obtained, the isolate was subjected to genotypic identification. Initially, identification with tRNA-PCR (13) in combination with capillary electrophoresis (1, 12) failed because no entries for *M. atlantae* were present in our tRNA-fingerprint library. However, since this *M. atlantae* isolate had tRNA spacer fragments with lengths of 116, 133, and 270 bp, a pattern which is different from all other members of the genus *Moraxella* and from those obtained for all bacterial species tested thus far, addition of this fingerprint to the library should enable future identification by means of tRNA-intergenic spacer-length polymorphism analysis. Two *M. atlantae* culture collection strains

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Region 1.

•190 •200 •210 •220

1. CGGGAGAAAGGAGCTTTT-----GGGTTCTGCTATAAGAT
 2. *****SYKWR-----A*S**Y*****
 3. *****GCGAG-----A*C**T*****
 4. *****A*****A-----*****
 5. *AA**CC**A**GGGGACCC*TCGG*CC*CT**C**CG**

Region 2.

•440 •450 •460 •470 •480

1. AAGCAGGGAGGAAGAGCCAGTGGAGAAACACACTGGT---GGTG
 2. *****T**CA*****GGG**A*---A***
 3. *****-----
 4. *****T**CA*****GGG**A*---A***
 5. C**G*****G*---**AA**T*TAC**TTGCTCAT**

FIG. 1. Alignment of two variable regions of the 16S rRNA gene sequences of four *M. atlantae* strains. IUB codes: S, C or G; Y, C or G; K, G or T; W, A or T; R, A or G. Sequences: 1, *M. atlantae* CCUG 10707 (GenBank no. AJ417491); 2, *M. atlantae* CCUG 31324 (GenBank no. AJ417492); 3, *M. atlantae* CDCA1922 (GenBank no. AF005191); 4, *M. atlantae* CCUG 45702, the clinical strain of this study (GenBank no. AJ313278); 5, *E. coli* ATCC 11775^T (GenBank no. X80725).

(CCUG 10707 and CCUG 31324) were shown to have tRNA spacer fragments with identical lengths.

16S rRNA gene amplification and sequencing was carried out as published before (11). Briefly, the complete 16S rRNA gene was amplified, followed by sequencing reactions using the Big Dye Terminator Sequencing kit (Applied Biosystems, Foster City, Calif.) and analysis of the obtained fragments on the ABI 310 capillary electrophoresis apparatus (Applied Biosystems). Total gene assembling of the obtained fragments, alignment, and clustering were done with GeneBase (Applied Maths, Kortrijk, Belgium). The obtained sequence (1,354 bp) was compared to all known sequences in the GenBank by Blast (National Center for Biotechnology Information, Bethesda, Md.; <http://www.ncbi.nlm.nih.gov/blast/index.html>). The Blast search resulted in a similarity of almost 99% with the only *M. atlantae* sequence present (GenBank number AF005191).

However, the sequences obtained for the *M. atlantae* strain of this study and for the GenBank *M. atlantae* strain contained two regions with substantial differences. Therefore, two *M. atlantae* culture collection strains (CCUG 10707 and CCUG 31324) were sequenced. This revealed other differences in the same regions. Those differences appeared to be nonrandom. For region 1 (*Escherichia coli* position 201 to 218), sequences a (TTTWGGGTTTC) and b (GCGAGAGCTTT) were observed. Sequence a was present in strain CCUG 10707 and the strain of this case report, and sequence b was observed for the GenBank entry AF005191. Strain CCUG 31324 apparently carried different alleles from either one or both sequences, since the ambiguities in the sequence obtained for this strain corresponded to a mixture of both sequences found in the other strains (Fig. 1). For region 2 (*E. coli* position 455 to 478), two possible sequences were seen, with strain CCUG 10707 and the GenBank entry AF005191 having one possible sequence and CCUG 31324 and our clinical isolate having the other. Other points of difference were found at *E. coli* position 381 (either C or G), *E. coli* position 668 (either A or G), *E. coli*

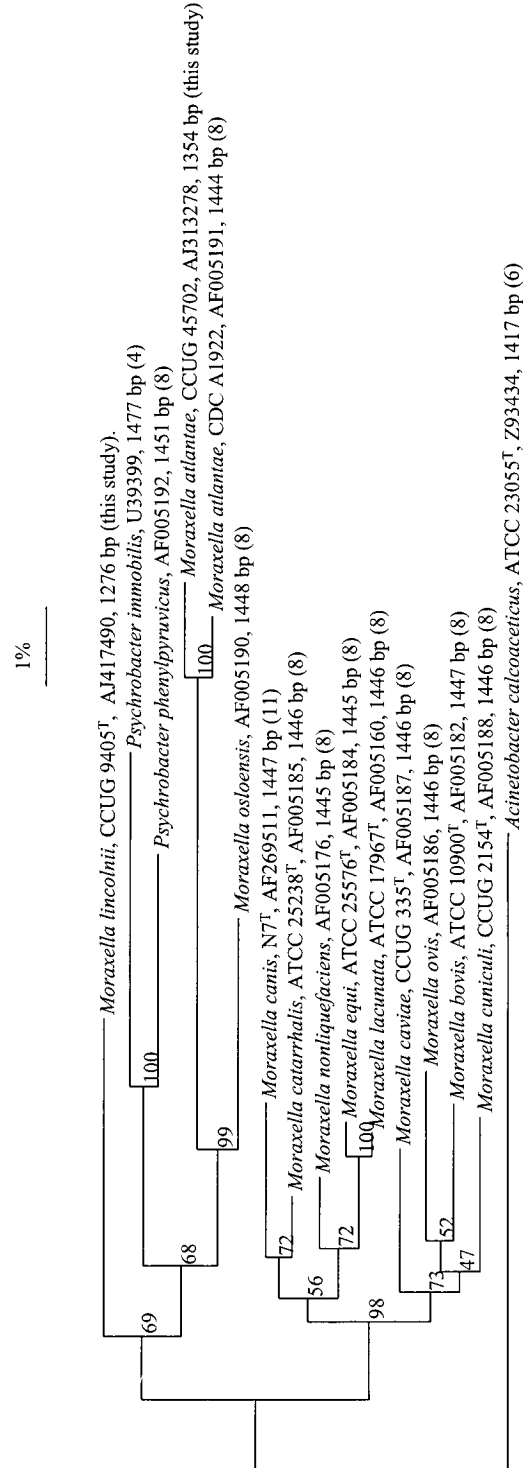


FIG. 2. Cluster analysis of 16S rRNA gene sequences of *M. atlantae* and other *Moraxellaceae*. Cluster analysis was based on the neighbor-joining method, with *A. calcoaceticus* as the outgroup. The bar indicates 1% homology divergence. The numbers in parentheses are references.

position 848 (either C or T), and *E. coli* position 1136 (either A or G).

In fact, all four strains have highly identical 16S ribosomal DNA sequences which at some regions are different mixtures of a few basic themes, probably reflecting past recombination events (5, 9). Furthermore, strain CCUG 31324 appears to have alleles with different sequences in a region between *E. coli* positions 201 and 218 whereby one or more cistrons have sequence a while one or more others have sequence b.

The 16S rRNA sequences of two *M. atlantae* isolates were compared with those of other moraxellae (including a more complete sequence of *Moraxella lincolnii* obtained in this study), *Psychrobacter immobilis* and *Psychrobacter phenylpyruvicus*, and the *Acinetobacter calcoaceticus* type strain (Fig. 2). *Psychrobacter* and *Acinetobacter* are genera which belong to the *Moraxellaceae* as well. A dendrogram of 16S rRNA gene sequences was constructed using the GeneBase software package. First, pairwise alignment using the unweighted pair group method with arithmetic mean was carried out with a gap penalty of 100%, a unit gap cost of 20%, and an ambiguity cost of 50% of the mismatch cost. Subsequently, global alignment with *A. calcoaceticus* as the outgroup was carried out on the region corresponding to positions 60 through 1334 of the 16S rRNA gene of *E. coli*, with costs as described above. Finally, a similarity matrix of the aligned sequences was constructed by global alignment homology calculation and a gap penalty of 20%. The neighbor-joining method was used to construct the dendrogram based on this similarity matrix (7).

This revealed that in fact the species *Moraxella osloensis*, *M. lincolnii*, and *M. atlantae* could be classified as separate genera, based on a less than 95% sequence relatedness to each other, to the other moraxellae, and to *Psychrobacter*. The similarity percentages were 91 for *M. atlantae* versus *M. lincolnii*, 92 for *M. atlantae* versus *M. osloensis*, 89 for *M. atlantae* versus the other moraxellae, 93 for *M. lincolnii* versus *M. osloensis*, and 89 for *M. atlantae* versus *P. immobilis* and *P. phenylpyruvicus*. The sequence differences between the two *Psychrobacter* species amounted to 5.7% as well. The suggestion that these species could be classified as separate genera is in correspondence to earlier reports based on 16S rRNA gene sequencing (8), DNA-DNA hybridization (10), and DNA transformation (2).

M. atlantae is an unusual and only rarely isolated bacterium, formerly known as CDC group M-3. Bøvre et al. (2) described five strains isolated from blood cultures, all with the tendency to spread on blood agar plates, but without mentioning the clinical importance. The strain of the only case report thus far (3) was isolated from a blood culture of a 25-year-old patient suffering from systemic lupus erythematosus and had the same biochemical and phenotypic characteristics as the strain from the present case report.

M. atlantae is a rare opportunistic pathogen that is apparently susceptible to most common antibiotics. This—together

with the difficulties encountered in most laboratories in the identification of gram-negative nonfermenters—may cause possible underestimation of its occurrence. For example, the Culture Collection of the University of Göteborg (CCUG) harbors 12 *M. atlantae* strains, of which 10 were isolated since 1981. Ten are from blood, one from pleural fluid, and one from a dog bite wound. Unambiguous identification of this organism is possible by means of 16S rRNA gene sequencing, tRNA-PCR, and phenotypic characteristics. Short, nonmotile, gram-negative rods forming small colonies, possessing twitching motility and corroding activity on blood agar, capable of growing on MacConkey agar, oxidase and catalase positive, not acidifying sugars, negative for nitrate reduction, urease, acetate assimilation, and tributyrin hydrolysis, and positive for pyrrolidone peptidase can be considered *M. atlantae*.

Nucleotide sequence accession numbers. The sequences of the 16S rRNA genes from *M. atlantae* and *M. lincolnii* obtained in this study were deposited under GenBank numbers AJ313278 and AJ417490, respectively.

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Mycobacterium interjectum as Causative Agent of Cervical Lymphadenitis

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A mycobacterial strain isolated from a lymph node of a 3-year-old female with cervical lymphadenitis was identified as *Mycobacterium interjectum* by means of sequencing of the 16S rRNA gene. Analysis of this case and previously published cases demonstrates the importance of *M. interjectum* as a causative agent of cervical lymphadenitis in young children.

Molecular techniques have made it possible to recognize previously overlooked mycobacterial species. A mycobacterial isolate from a lymph node of a child with lymphadenitis could not be identified with amplified ribosomal DNA restriction analysis (ARDRA), the molecular technique routinely used at our laboratory for identification of mycobacteria. Determination of the sequence of the 16S rRNA gene led to a final identification as *Mycobacterium interjectum* and prompted us to review previously described cases of infection due to this organism.

Case report. A 3-year-old female was admitted to Ghent University Hospital with cervical lymphadenitis lasting for a period of 8 weeks. In the left submandibular and left parotid areas, a firm nodular mass of 3 by 4 cm was palpable and the overlying skin was blue-red. There was discrete local pain but no systemic illness. A chest X-ray and routine hematological examination were normal. An intradermal skin test using purified protein derivative (PPD) (five tuberculin units) of both tuberculous and nontuberculous mycobacteria was applied, and an induration with a diameter of more than 10 mm was seen for *M. avium* complex. Because antibiotic treatment with azithromycin given for 3 weeks resulted in no response, complete surgical excision was performed.

During surgery, the affected skin was resected, including a left parotidectomy and resection of the submandibular gland with associated lymph nodes in the superior jugular area. Samples of the lymph nodes were preserved for laboratory investigation and culturing. Diffuse tuberculoid granulomatous lymphadenitis and caseous necrosis were seen throughout the specimen. *M. interjectum* was isolated from all resected lymph nodes. Drug susceptibility testing was performed by the proportion method with Lowenstein-Jensen (LJ) medium or Middlebrook 7H11 agar (7H11) and a single concentration of each drug as recommended before (1, 7). The isolate was found to be resistant to rifampin (40 µg/ml of LJ), isoniazid (0.2 µg/ml of LJ), ethambutol (2 µg/ml of LJ), *para*-aminosal-

icylic acid (0.5 µg/ml of LJ), streptomycin (4 µg/ml of LJ), kanamycin (6 µg/ml of 7H11), and capreomycin (10 µg/ml of 7H11) and susceptible to cycloserine (60 µg/ml of 7H11), ethionamide (10 µg/ml of 7H11), clarithromycin (1 µg/ml of LJ), rifabutin (40 µg/ml of LJ), and ofloxacin (4 µg/ml of 7H11).

After surgery, the patient recovered completely and no relapse was seen.

Preparation of the specimens for mycobacterial culturing started with *N*-acetyl-L-cysteine-NaOH-based decontamination, followed by auramine staining and inoculation of a liquid medium (MB BacT; Organon Teknika, Bostel, The Netherlands) and a solid medium (Ogawa; Sanofi-Pasteur, Marnes-la-Coquette, France). The auramine staining (auramine obtained from Merck, Darmstadt, Germany) was negative, no growth was observed on the solid medium, but the liquid culture became positive after 30 days.

At our laboratory, identification of cultured mycobacteria is done by ARDRA (14), which consists of restriction digestion of the amplified 16S rRNA gene. For the isolate obtained here, the combination of *Cfo*I restriction pattern 5, *Mbo*I pattern 4, and *Rsa*I pattern 4 was observed; this profile did not correspond to any of the profiles of the mycobacterial species included in our reference panel (14; <http://allserv.rug.ac.be/~mvaneech/ARDRA/Mycobacterium.html>). Therefore, sequencing of the 16S rRNA gene was necessary to obtain final identification. Sequencing was carried out as described previously (6). The obtained sequence was compared with all known sequences of GenBank by use of Blast 2.0 (National Center for Biotechnology Information, Bethesda, Md. [<http://www3.ncbi.nlm.nih.gov/BLAST/>]) and showed 99.8% similarity with *M. interjectum*. Identification as *M. interjectum* was confirmed by cluster analysis performed by use of Genecompar (Applied Maths, Kortrijk, Belgium). The sequences of the following strains were used in the UPGMA (unweighted pair-group method using arithmetic averages) clustering: Ghent University Hospital clinical strain (GenBank accession no. AJ272088), four previously sequenced *M. interjectum* strains (GenBank accession no. AF014935, AF014936, AF014937, and X70961), an *M. simiae* strain (GenBank accession no. X52931), an *M. heidelbergense* strain (GenBank accession no. AJ000684) (in the latter two species, the 16S rRNA gene sequence clusters

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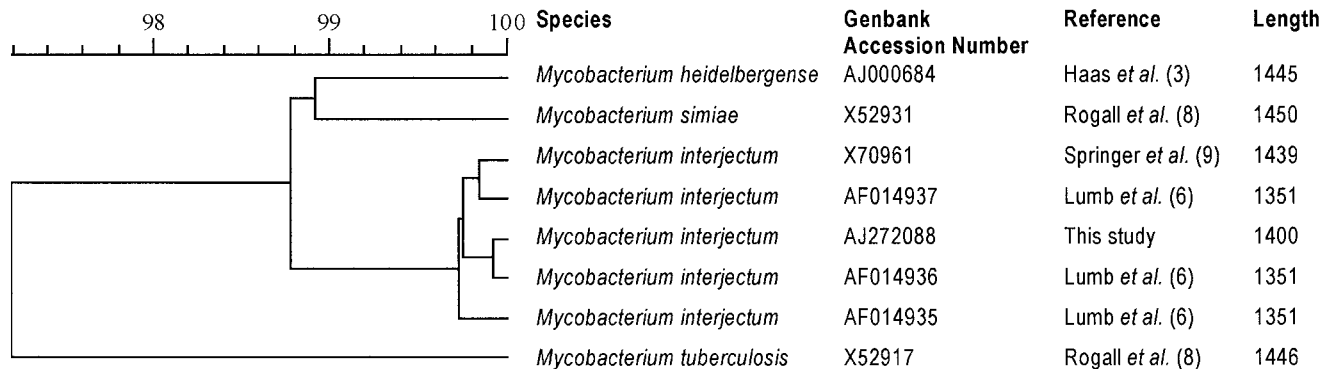


FIG. 1. UPGMA clustering of the 16S rRNA gene sequence of the clinical isolate reported in this study with sequences obtained from GenBank. Lengths are shown in base pairs.

very closely with that of *M. interjectum*), and an *M. tuberculosis* strain (GenBank accession no. X52917) (Fig. 1).

Further confirmation of the sequencing results was done by biochemical testing as described before (15). The strain was found to be a scotochromogenic, slowly growing mycobacterium, susceptible on LJ medium to NaCl (5%) and isoniazid (10 µg/ml) but resistant to carboxylic acid hydrazide (2 µg/ml), hydroxylamine (250 µg/ml), and *para*-nitrobenzoic acid (500 µg/ml).

The strain was found to be negative for semiquantitative catalase (i.e., less than 45-mm foam production) nitrate reduction, acid phosphatase, and niacin production but positive for Tween hydrolysis and urease. Thin-layer chromatography of the fatty acids revealed alpha-, methoxy-, and keto-mycolic acids. All of these biochemical characteristics fit with the identification as *M. interjectum* (10). The negative catalase reaction, the positive Tween hydrolysis, and the susceptibility to 10 µg of isoniazid per ml, as well as the mycolic acid pattern, differen-

tiate this species from the phenotypically very similar species *M. scrofulaceum* (15).

M. interjectum, for which the species name refers to the intermediate phylogenetic position between rapidly and slowly growing mycobacteria, was first described in 1993 (9). Table 1 summarizes the clinical features of all published cases in which *M. interjectum* was isolated.

M. interjectum was described as the causative agent in five pediatric cases of cervical lymphadenitis. Four cases in adults have been described, but in only one case (Table 1, case 7) was *M. interjectum* considered clinically important. The four patients (three pediatric) treated with antibiotics alone and/or undergoing partial resection were not cured. Cure was obtained only after total resection of the infected region.

Nontuberculous mycobacterial lymphadenitis was traditionally associated with *M. scrofulaceum* (5). During the 1980s, the *M. avium* complex was predominant (16). More recently, however, a wide variety of mycobacterial species causing lymphad-

TABLE 1. Summary of the clinical features of our case and previously reported cases of infection with *M. interjectum*

Case (reference)	Patient age/sex	Symptoms	Treatment (initial; subsequent)	Outcome (initial; subsequent)
1 (this study)	3 yr/female	Cervical swelling in the left submandibular region	Azithromycin; total resection	No response; no relapse
2 (9)	18 mo/male	Enlarged lymph node in the right submandibular region	Partial resection + isoniazid, rifampin, and pyrazinamide; Total resection + clarithromycin, isoniazid, and protionamid	Further enlargement of the lymph node, including a fistula; no relapse
3 (12)	2 yr/female	Right laterocervical swelling	Clarithromycin; total resection	No response; no relapse
4 (6)	2 yr/female	Enlarged lymph node in the left anterior triangle	Total resection	No relapse
5 (6)	3 yr/female	Cervical swelling in the left anterior triangle and mildly enlarged node in the right neck	Total resection	No relapse
6 (6)	Elderly/female	Widespread interstitial lung opacities	No treatment (considered not clinically significant)	
7 (2)	52 yr/female	Chronic destructive lung disease, with multiple positive cultures	Amikacin	No response
8 (11)	71 yr/female	No specific symptoms; a single isolate from a urine sample	No treatment (considered not clinically significant)	
9 (10)	36 yr/male	No specific symptoms; a single isolate from sputum of an AIDS patient	No treatment (considered not clinically significant)	

enitis in young children have been reported, including some previously unrecognized mycobacteria (3, 4, 9, 12, 13). At present, it is difficult to establish whether this observation reflects real changes in the prevalence of different mycobacterial species or is due to increased diagnostic capabilities and to refined mycobacterial taxonomy.

Full identification of the nontuberculous agents causing cervical lymphadenitis in young children is warranted to reveal the role of different mycobacterial species and may indicate an underestimation of the pathogenic role of species such as *M. interjectum*. Also, correct identification may be important to guide therapy, since present experience seems to indicate that total resection is the only cure for lymphadenitis caused by species such as *M. interjectum*. Identification of *M. interjectum* by phenotypic methods is slow and not always straightforward, since the species has been reported to have quite a few variable reactions (6). Also, with high-pressure liquid chromatography analysis of mycolic acids, differences among the patterns obtained for different *M. interjectum* strains have been reported (6, 10). Accurate identification of *M. interjectum* is possible by means of 16S rRNA gene sequencing and by means of ARDRA, which results in *CfoI* restriction pattern 5, *MboI* pattern 4, and *RsaI* pattern 4, a profile thus far observed only for *M. interjectum*.

We thank Leen Van Simaey for excellent technical assistance.

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Classification of *Ralstonia pickettii* biovar 3/ 'thomasi' strains (Pickett 1994) and of new isolates related to nosocomial recurrent meningitis as *Ralstonia mannitolytica* sp. nov.

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Strains isolated independently from two patients could be recognized as *Ralstonia pickettii* biovar 3/ 'thomasi'. The 16S rDNA sequences of these strains and two other strains of *R. pickettii* biovar 3/ 'thomasi' clustered at less than 98% similarity versus all other described *Ralstonia* species and at less than 97% versus the two other *R. pickettii* biovars. The separate species status of *R. pickettii* biovar 3/ 'thomasi' was confirmed by DNA–DNA hybridization, indicating less than 60% DNA homology with the *R. pickettii* biovars Va-1 and Va-2 and with two as-yet unclassified but biochemically similar *Ralstonia* strains. Phenotypically, this *Ralstonia* species can be distinguished from all described *Ralstonia* species by its acidification of D-arabitol and mannitol and by its lack of nitrate reduction and of alkalization of tartrate and from two as-yet unclassified *Ralstonia* strains only by its lack of nitrate reduction. The name *Ralstonia mannitolytica* sp. nov. is proposed, reflecting the characteristic acidification of mannitol. Resistance to desferrioxamine is another difference from *R. pickettii* and *Ralstonia solanacearum*. Although several nosocomial outbreaks have been associated with *R. mannitolytica*, life-threatening infections have not yet been reported, possibly due to misidentification as *Pseudomonas fluorescens* or *Burkholderia cepacia*. In at least one of the two cases reported here, the *R. mannitolytica* isolate was found to be clinically relevant, causing recurrent nosocomial meningitis, with an infected implanted catheter as the source. The type strain of *R. mannitolytica* is NCIMB 10805^T (= LMG 6866^T), which was isolated during the first described outbreak as '*Pseudomonas thomasi*' at St Thomas' Hospital, London, UK, in 1971.

Keywords: *Ralstonia pickettii* biovar 3/ 'thomasi', *Ralstonia mannitolytica* sp. nov., infection

INTRODUCTION

The genus *Ralstonia* (Yabuuchi *et al.*, 1995) has been created for a group of organisms from ecologically diverse niches to accommodate bacteria that were formerly classified as members of *Burkholderia*

(Yabuuchi *et al.*, 1992) and *Alcaligenes*. The type species of the genus, *Ralstonia pickettii*, was originally regarded as the only representative of clinical importance (Dimech *et al.*, 1993; Fass & Barnishan, 1976; Fujita *et al.*, 1981; Gardner & Shulman, 1984; Kahan *et al.*, 1983; Lacey & Want, 1991; Raveh *et al.*, 1993; Roberts *et al.*, 1990; Verschraegen *et al.*, 1985). Only recently, the taxonomy of this genus has begun to be elucidated with the description of several new species, *Ralstonia paucula*, *Ralstonia gilardii* and *Ralstonia basilensis*, some of which are of moderate

Abbreviation: TSA, tryptic soy agar.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences reported in this paper are AJ270252–AJ270266 and AJ271437.

clinical importance (Coenye *et al.*, 1999; Vandamme *et al.*, 1999; Moissenet *et al.*, 1999; Osterhout *et al.*, 1998; Steinle *et al.*, 1998).

Historically, *R. pickettii* was created for a group of clinical isolates (Ralston *et al.*, 1973) and also turned out to include strains of CDC group Va-2 (Tatum *et al.*, 1974; Riley & Weaver, 1975) and possibly other strains. The CDC groups Va-2 and Va-1 were regarded as two different biovars and hence Pickett & Greenwood (1980) decided that CDC groups Va-1 and Va-2 represented two different biovars of *R.* (*Pseudomonas*) *pickettii*. Shortly before this, King *et al.* (1979) had concluded that *R.* (*Pseudomonas*) *pickettii* contained several biovars, including the strains originally isolated from clinical samples in St Thomas' Hospital, London, UK (Phillips & Eykyn, 1972; Phillips *et al.*, 1972), and which had been designated '*Pseudomonas thomasi*'. Although this name was never validated, it has been used several times (Baird *et al.*, 1976; King *et al.*, 1979; Costas *et al.*, 1990). According to Pickett (1994), the present taxonomic situation of *R. pickettii* should be reduced to the recognition of three biovars, with biovar 1 equal to CDC group Va-1, biovar 2 equal to CDC group Va-2 and biovar 3 mostly consistent with the invalid '*Pseudomonas thomasi*' (Pickett, 1994).

Here, we report a case of recurrent nosocomial meningitis caused by a colistin-resistant, Gram-negative rod that was identified by means of API 20NE as *Pseudomonas fluorescens*. Because of its colistin resistance, this strain was studied in more detail, which lead to its identification as *R. pickettii* biovar 3/'*thomasi*'. The further data gathered, by means of sequence determination of the 16S rDNA, determination of the cellular fatty acid composition, DNA hybridization, SDS-PAGE and extensive characterization of biochemical reactivity, indicate that this biovar can be considered as a separate species within the genus *Ralstonia*, named *Ralstonia mannitolytica* sp. nov. after its characteristic acidification of mannitol, distinct from all other described *Ralstonia* species. Retrospectively, a strain stored previously as *P. fluorescens* and isolated repeatedly from an abdominal haematoma of a patient with cholangiocarcinoma and a strain from an outbreak due to contaminated water in the neonatal intensive care department of the Ghent University Hospital, Belgium (GUH), in 1990 could also be identified as *R. mannitolytica*. Several nosocomial outbreaks with *R. pickettii* biovar 3/'*thomasi*', mostly due to contaminated fluids, have been described (Costas *et al.*, 1990; Pan *et al.*, 1992; Phillips *et al.*, 1972), but no life-threatening infections have been reported yet to our knowledge.

METHODS

Bacterial strains. The strains studied are listed in Table 1. The clinical strains LMG 19090, LMG 19091 and LMG 19092 were isolated at the GUH. Strain LMG 19090 (LBV407/UCL310) was isolated repeatedly from a patient with

recurrent meningitis (patient 1), strain LMG 19091 (LBV371/RAL13) was isolated repeatedly from an abdominal haematoma of a patient with cholangiocarcinoma (patient 2) and strain LMG 19092 (PSE061) was one of several strains isolated during an outbreak due to contaminated water at the GUH neonatal intensive care department in 1990. Strains LMG 19083 (RAL05/ML7), LMG 19087 (RAL07/YL13), LMG 19088 (RAL06/ML10) and LMG 19089 (RAL04/MC5) are environmental soil isolates described by Suyama *et al.* (1998) and were included because some of these strains showed the highest 16S rDNA sequence similarity to the clinical strains of all the sequences available from the GenBank/EMBL database. All other strains studied here, including the *R. mannitolytica* type strain (LMG 6866^T), were supplied by the BCCM/LMG Bacteria Collection (Ghent, Belgium) or were isolated at the GUH. [*Pseudomonas*] *syzygii* strains, which are so closely related to *Ralstonia solanacearum* (Brim *et al.*, 1998; this study) that they can be regarded as members of this genus, were included to exclude synonymy with the *R. pickettii* biovar 3/'*thomasi*' strains studied here.

Biochemical characterization. Growth at 30 and 37 °C was evaluated on tryptic soy agar (TSA) (Sanofi Diagnostics Pasteur). Growth at 42 °C was tested by adding two drops of a McFarland suspension 3 in water, prepared from a 24 h culture on TSA, to 5 ml of tryptic soy broth and subsequent incubation in a water bath at 42 °C and reading after 48 h. Conventional tests were carried out as described elsewhere (Gilligan, 1995). Commercial tests (API ZYM, API 20NE, API 50CH and ID 32GN; bioMérieux) were carried out according to the instructions of the manufacturer. Assimilation testing was carried out using the AUX medium (bioMérieux). To test for assimilation and alkalization of organic substrates, Simmons' citrate agar was used, whereby citrate was replaced by organic substrates at a concentration of 0.2% (w/v). Desferrioxamine susceptibility was tested according to a method adapted from Lindsay & Riley (1991), by loading 6 mm diameter paper discs with 250 µg desferal on Müller Hinton II agar (MHA) (BBL Becton Dickinson). Alkaline phosphatase, pyrrolidonyl arylamidase and benzyl arginine arylamidase (trypsin) were tested using Rosco tablets (Taastrup, Denmark) and read after 4 h. Colistin susceptibility was tested using 10 µg colistin paper disks (BBL) on MHA and susceptibility to the vibriostatic agent O:129 was tested with Sanofi Diagnostics Pasteur paper discs on MHA. Acidification of ethylene glycol was tested as described previously (Wauters *et al.*, 1998). Staining of flagella was done as described previously (Kodaka *et al.*, 1982).

16S rDNA sequence determination. Amplification was done by PCR with primers named α/β NOT (5'-AGTTTGATCCTGGCTCAG-3') and ω MB (5'-TACCTTGTTACGACTTCGTCCCA-3'). The reactions were performed in a final reaction mixture of 50 µl containing 25 µl Master Mix (Qiagen), 0.2 µM of each primer and 5 µl of a DNA suspension obtained by alkaline lysis. The amplification reactions were performed in a GeneAmp PCR System 9600 (Perkin-Elmer Applied Biosystems) with the following cycling parameters: 94 °C for 5 min, followed by three cycles of 45 s at 94 °C, 2 min at 50 °C and 1 min at 72 °C and 30 cycles of 20 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C, with a final extension at 72 °C for 7 min. The amplification products were checked by 2% agarose gel electrophoresis and staining with ethidium bromide. The amplification products were purified with a PCR purification kit (Qiagen) according to the manufacturer's instructions. Sequencing

Table 1 List of strains studied

Names of taxa in brackets indicate that the organisms were misnamed at the corresponding taxonomic level. Abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; CDC, Centers for Disease Control, Atlanta, GA, USA; CIP, Collection Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; GUH, Ghent University Hospital, Belgium; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; NCIB, National Collection of Industrial Bacteria, Aberdeen, UK; NCPPB, National Collection of Plant-pathogenic bacteria, Harpenden Laboratory, Herts, UK; UCL, Université Catholique de Louvain, Belgium.

Strain	Other strain designation(s)	Origin
[<i>P.</i>] <i>syzygii</i>		
LMG 6969	RAL08, Eden-Green S593	
LMG 6970	RAL09, Eden-Green S594	
LMG 10661 ^T	RAL10 ^T , Eden-Green R001 ^T , Jones W1330 ^T , NCPPB 3446 ^T	West Sumatra (' <i>Syzygium aromaticum</i> ')
LMG 10662	RAL11, Eden-Green R002, NCPPB 3445	West Java
<i>R. basilensis</i>		
LMG 18990*	DSM 11853, Steinkle RK1	Laboratory fixed bed reactor, 2,6-dichlorophenole as sole energy source
<i>R. eutropha</i>		
LMG 1194	RAL14, CCUG13796, DSM 515, Schlegel B19	USA, forest soil
LMG 1199 ^T	RAL15 ^T , ATCC 17697 ^T , CCUG 1776 ^T , DSM 531 ^T , ICPB 3984 ^T , NCIB 11842 ^T	USA, soil
LMG 1201	RAL16, ATCC 17699, ATCC 23440, CCUG 13797, DSM 428, NCIB 10442, Schlegel H16	Göttingen, Germany, source sludge
<i>R. gilardii</i>		
LMG 5886 ^T *	API 141-2-84 ^T , ATCC 700815 ^T , Gilardi 4325 ^T	Whirlpool
<i>R. mannitolytica</i>		
LMG 6866 ^T	NCIB 10805 ^T	St. Thomas' Hospital, London, UK, 1971, blood
LMG 19090	LBV407, UCL310	GUH, 1998, recurrent meningitis
LMG 19091	RAL13, LBV371, GUH 98 06 3523	GUH, 1998, abdominal haematoma
LMG 19092	PSE061, GUH S90 03985	GUH, 1990, sputum
<i>R. paucula</i>		
LMG 3244 ^T	ATCC 700817 ^T , CCUG 12507 ^T , CDC E6793 ^T	Oregon, USA, human respiratory tract
LMG 3515	CCUG 12411, CCUG 12505, CDC E8967, LMG 3245	Georgia, USA, sputum
<i>R. pickettii</i>		
LMG 7001*	CCUG 3314, Pickett K-214	USA, Sephadex eluate
LMG 7002*	CCUG 3316, LMG 7146, Pickett K-279	
LMG 7008*	CCUG 2165	Göteborg, Sweden, 1973, urine
LMG 7145*	CCUG 3315, Pickett K-232	
<i>R. pickettii</i> biovar Va-1		
LMG 7005	CCUG 1467, CDC A5832, PSE056	
LMG 7014	RAL02, CCUG 12491, Gilardi GLG 3963	Blood
LMG 7015	RAL03, CCUG 12492, Gilardi GLG 3905	Blood
LMG 7160	RAL01, CIP 74.22, CIP R866	Blood culture
LMG 19083	ML7, RAL05	Soil, Japan
LMG 19084	PSE058, GUH S89 04031	GUH, 1989, sputum
LMG 19086	PSE060, GUH S89 04266	GUH, 1989, sputum
LMG 19088	ML10, RAL06	Soil, Japan
<i>R. pickettii</i> biovar Va-2		
ATCC 27512	WG076, Pickett K-550	Patient with tracheotomy, 1973
LMG 5942 ^T	ATCC 27511 ^T , CCUG 3318 ^T , CIP 73.23 ^T , ICPB 3981 ^T , JCM 5969 ^T , NCTC 11149 ^T , Pickett K-288 ^T , PSE055 ^T , WG071 ^T	Los Angeles, CA, USA, patient with tracheotomy
LMG 19085	RAL12, GUH 99 03 4045	GUH, 1999, nebulizer
<i>R. solanacearum</i>		
LMG 2299 ^T	RAL17 ^T , CCUG 14272 ^T , DSM 9544 ^T , JCM 10489 ^T , Kelman 60-1 ^T , NCPPB 325 ^T	USA, <i>Lycopersicon esculentum</i>
LMG 2303	RAL18, NCPPB 078	Honduras
<i>Ralstonia</i> sp.		
LMG 19087	YL13, RAL07	Japan, soil
LMG 19089	MCS, RAL04	Japan, soil

* Strain was only included in SDS-PAGE of whole-cell proteins.

was performed on an ABI 310 sequencer using the ABI Big Dye cycle sequencing reaction kit with AmpliTaq FS DNA polymerase (Perkin Elmer) with primers described previously (Coenye *et al.*, 1999). Analysis of the sequences and clustering was done by using GENECOMPARE version 4.1 (Applied Maths).

DNA base composition. DNA was degraded enzymatically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters Symmetry Shield C8 column thermostabilized at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH

4.0) with 1.5 % acetonitrile. Non-methylated lambda phage DNA (Sigma-Aldrich) was used as the calibration reference.

DNA-DNA hybridizations. DNA-DNA hybridizations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements (excitation filter of 360 nm, emission filter of 465 nm). The optimal renaturation temperature was determined according to the equation of De Ley *et al.* (1970).

PAGE of cell proteins. Cells were grown for 48 h on TSA at 37 °C. SDS protein extracts were prepared and electro-

Table 2 Phenotypic characteristics useful in the differentiation of the *Ralstonia* species

The following taxa were included in the analysis: 1, *R. mannitolytica* strains LMG 6866^T, LMG 19090, LMG 19091 and LMG 19092; 2, *R. pickettii* biovar Va-1 strains LMG 7014, LMG 7015, LMG 7160, LMG 19083, LMG 19084, LMG 19086 and LMG 19088; 3, *R. pickettii* biovar Va-2 strains ATCC 27512, LMG 5942^T and LMG 19085; 4, *Ralstonia* sp. strain LMG 19089; 5, *Ralstonia* sp. strain LMG 19087; 6, *R. solanacearum* strains LMG 2299^T and LMG 2303; 7, *R. eutropha* strains LMG 1194, LMG 1199^T and LMG 1201; 8, *R. paucula* strains LMG 3515 and LMG 3244^T; and 9, *R. gilardii*. Data for *R. gilardii* were taken from Coenye *et al.* (1999). R, Resistant; s, susceptible; NK, not known. Growth is scored as: v, variable; +, all strains positive; (+), weakly positive; -, all strains negative.

Characteristic	1	2	3	4	5	6	7	8	9
Flagellation	Polar 1	Polar 1	Polar 1	Polar 1	Polar 1	None	Peritrichal	Peritrichal	Polar 1
Reaction to:									
Colistin	R	R	R	R	R	R	S	S	NK
Desferrioxamine	R	S	S	R	R	S	R	R	NK
Viability on TSA at room temperature	< 6 d	< 6 d	< 6 d	< 6 d	< 6 d	< 6 d	> 21 d	> 21 d	NK
Growth at 42 °C	+	v	v	+	-	-	v	v	+
Catalase	+	+	+*	+	+	+	+	+	+
Oxidase	+	+*	+*	+	+	+	+	+	-
Alkaline phosphatase (Rosco)	-	-	-	-	-	-	+	-†	+‡
Pyrrolidonol arylamidase	+	+	+	+	+	-	+	+	NK
Phenylalanine deaminase	v§	v§	+	v§	-	-	v	-	NK
Urease	+	+	+	+	+	+	-	+	-
Nitrate reduction	-	+	+	+	+	+	v	-	-
Tween 80 hydrolysis	+	+	+	+	+	+	v	+	NK
Production of acid from:									
l-Arabinose	+	+	+	+	+	-	-	-	-
D-Arabitol	+	-	-	+	+	-	-	-	NK
Glucose	+	+	+	+	+	+	-	-	NK
Inositol	-	-	-	-	-	+	-	-	NK
Lactose	+	+	-	+	+	-	-	-	NK
Maltose	+	+	-	+	+	-	-	-	NK
Mannitol	+	-	-	+	+	-	-	-	NK
Sucrose	-	-	-	-	-	+	-	-	NK
D-Xylose	+	+	+	+	+	-	-	-	-
Alkalinization of:									
Acetate	+	+	+	+	+	-	+	+	NK
Allantoin	-	-	-	-	-	-	+	-	NK
Citrate	+	+	+	+	+	+	+	+	-
Galacturonate	+	+	+	+	+	+	-	-	NK
Lactate	+	+	+	+	+	-	+	+	NK
Maleate	-	+	+	-	(+)	v	v	v	NK
Malonate	+	+	+	+	+	-	+	+	NK
Mucate	+	+	+	+	+	+	-	+	NK
Oxalate	-	-	-	-	-	-	+	+	NK
Tartrate	-	+	+	-	-	+	+	+	NK
Assimilation of:									
3-Hydroxybenzoate	-	-	-	-	-	-	+	-	NK
Caprate	+	+	+	+	+	-	+	+	-
Propionate	+	+	+	+	+	-	+	+	NK
Suberate	+	+	+	+	+	-	+	+	NK

* Negative according to Vandamme *et al.* (1999).

† Negative with Rosco tablets, containing nitrophenyl phosphate as the substrate. Vandamme *et al.* (1999) reported a positive reaction, using API ZYM (naphthyl phosphate as the substrate). In this study, all strains tested with API ZYM were positive.

‡ Tested with API ZYM.

§ Phenylalanine deaminase reactions were difficult to interpret (weakly positive) and very variable.

phoresed according to Pot *et al.* (1994) and the collected data were interpreted as described by Vauterin & Vauterin (1992). The similarity between all pairs of electrophoresis patterns was calculated by the Pearson product-moment correlation coefficient, expressed as a percentage.

Determination of cellular fatty acid composition. Cellular fatty acids were extracted from cells grown for 48 h on TSA at 37 °C. The fatty acid analysis was performed at 45–210 °C by using a capillary column CP-Sil 88 (Chrompack International) and a Delsi DI 200 chromatograph (Intersmat) equipped with split-splitless injection and a flame-ionization detector. The results were expressed as relative percentages

by using an Enica 31 integrator obtained from Delsi Nermag Instruments (Intersmat).

RESULTS

Microbiology

Strain LMG 19090, obtained from patient 1, was isolated on *Burkholderia cepacia* selective medium (Mast Diagnostics), containing 100 mg ticarcillin l⁻¹ and 300 U polymyxin B ml⁻¹. Testing with API 20NE resulted initially in identification as *P. fluorescens*

Table 3 Cellular fatty acid composition (mol %)

Taxon	Strains	16:1 ^{9cis}	16:0	18:1 ^{11cis}	14:0
<i>R. mannitolytica</i>	LMG 6866 ^T , LMG 19090, LMG 19091	30–38	21–30	7–15	5–7
<i>R. pickettii</i> biovar Va-1	LMG 7160, LMG 7014, LMG 19084	41–42	26–28	13–15	10
<i>R. solanacearum</i>	LMG 2299 ^T , LMG 2303	41–42	26–33	9–13	6–10
<i>R. eutropha</i>	LMG 1194, LMG 1199 ^T , LMG 1201	41–46	27–30	14–18	4–6
<i>R. paucula</i>	LMG 3515, LMG 3244 ^T	42–44	22–23	17–20	6–7

Table 4 DNA G + C content and DNA–DNA binding values of strains examined

Taxon	G + C content (mol%)	DNA–DNA binding with DNA from (%):								
		1	2	3	4	5	6	7	8	9
1. <i>R. mannitolytica</i> LMG 6866 ^T	66.2	100								
2. <i>R. mannitolytica</i> LMG 19090	66.2	88	100							
3. <i>R. mannitolytica</i> LMG 19091	66.2	86	100	100						
4. <i>R. pickettii</i> biovar Va-2 LMG 5942 ^T	64.0	58	ND	ND	100					
5. <i>R. pickettii</i> biovar Va-2 LMG 19085	64.1	53	48	52	85	100				
6. <i>Ralstonia</i> sp. LMG 19089	65.1	56	58	57	64	64	100			
7. <i>R. pickettii</i> biovar Va-1 LMG 19083	64.1	46	ND	ND	57	62	66	100		
8. <i>R. pickettii</i> biovar Va-1 LMG 19088	64.1	50	ND	ND	61	61	68	100	100	
9. <i>Ralstonia</i> sp. LMG 19087	64.0	50	ND	ND	63	63	71	65	69	100

ND, Not done.

(profile code 0 054 555). Strain LMG 19091, obtained from patient 2, had been identified previously as *P. fluorescens* (API profile code 0 045 555). Strain LMG 19092 (PSE061) was isolated in 1990 from the sputum of a neonate during an outbreak in the paediatric department and had been identified at that time as *R. pickettii*. The combined features of biochemical characteristics and colistin resistance indicated that these isolates were strains of *R. mannitolytica* sp. nov. (formerly *Ralstonia pickettii* biovar 3/‘*thomasii*’) (Clark *et al.*, 1984; Gilligan, 1995).

The biochemical characteristics useful for the differentiation of *Ralstonia* species are summarized in Table 2. The three clinical *R. mannitolytica* strains were motile by a single polar flagellum, while motility was not observed for the culture collection *R. mannitolytica* type strain LMG 6866^T. During this study, it was observed that freshly isolated strains were very motile and that motility decreased upon prolonged preservation and subculture. The type strain of *R. mannitolytica*, LMG 6866^T, was non-motile, possibly as a consequence of prolonged preservation.

All four strains grew at 30, 37 and 42 °C and were viable for less than 6 d on TSA at room temperature. Oxidase and catalase were positive. They were resistant to desferrioxamine, O:129 and colistin. No acid was produced from ethylene glycol. Urease, pyrrolidonyl arylamidase (Rosco), Tween esterase and phenylalanine deaminase were positive. Nitrate reduction,

indole and hydrogen sulfide production, alkaline phosphatase (Rosco), arginine dihydrolase, lysine and ornithine decarboxylases and aesculin and gelatin hydrolysis were negative. Acid was produced oxidatively from glucose, L-arabinose, lactose, maltose, mannitol, D-arabitol and D-xylose. Alkalinization occurred on minimal mineral agar with acetate, serine, malonate, β-alanine, 4-aminobutyrate, azelate, succinate, fumarate, butyrate, formate, malate, mucate, galacturonate, citrate, histidine and lactate, but not with acetamide, adipate, alginate, allantoin, amygdalin, L-arginine, benzoate, L-ornithine, maleate or tartrate.

Testing by means of API 20NE, API 50CH and ID 32GN indicated that the strains assimilated acetate, N-acetylglucosamine, L-alanine, L-arabinose, D-arabitol, 4-hydroxybenzoate, 3-hydroxybutyrate, caprate, citrate, fructose, galactose, gluconate, glucose, 2-keto-gluconate, glycerol, histidine, D-lactate, malate, malonate, mannitol, L-proline, propionate, serine, suberate and D-xylose, but not adipate, amygdalin, L-fucose, glycogen, inositol, itaconate, 5-ketogluconate, maltose, mannose, melibiose, phenylacetate, rhamnose, ribose, sucrose, salicin, D-sorbitol or 3-hydroxybenzoate and confirmed the absence of nitrate reduction.

Using the API ZYM system, the following enzymes were detected: C4 esterase, C8 esterase-lipase, C14 lipase, leucine arylamidase, acidic and alkaline phos-

Table 5 16S rDNA sequences included in this study

Taxon	16S rDNA accession no.	No. of nucleotides sequenced	Reference(s)
[<i>P.</i>] <i>syzygii</i>	AB021403	1513	–
[<i>P.</i>] <i>syzygii</i> LMG 10661 ^T	AJ270252	469	This study
[<i>P.</i>] <i>syzygii</i> LMG 6970	AJ270253	464	This study
[<i>P.</i>] <i>syzygii</i> LMG 10662	AJ270254	463	This study
[<i>P.</i>] <i>syzygii</i> LMG 6969	AJ270255	449	This study
<i>R. eutropha</i>	M32021	1511	–
<i>R. gilardii</i>	AF076645	1451	Coenye <i>et al.</i> (1999)
<i>R. mannitolytica</i> LMG 19091	AJ270256	1453	This study
<i>R. mannitolytica</i> LMG 19090	AJ270257	1450	This study
<i>R. mannitolytica</i> LMG 19092	AJ271437	517	This study
<i>R. mannitolytica</i> LMG 6866 ^T	AJ270258	1449	This study
<i>R. paucula</i>	AF085226	1484	Vandamme <i>et al.</i> (1999)
<i>R. pickettii</i> bv. Va-1 LMG 7160	AJ270260	1450	This study
<i>R. pickettii</i> bv. Va-1 LMG 19083	AB013428	441	This study, Suyama <i>et al.</i> (1998)
<i>R. pickettii</i> bv. Va-1 LMG 19088	AB013429	447	This study, Suyama <i>et al.</i> (1998)
<i>R. pickettii</i> bv. Va-1 LMG 19084	AJ270261	342	This study
<i>R. pickettii</i> bv. Va-1 LMG 7015	AJ249848	458	This study
<i>R. pickettii</i> bv. Va-1 LMG 7005	AJ270262	424	This study
<i>R. pickettii</i> bv. Va-1 LMG 7014	AJ270264	472	This study
<i>R. pickettii</i> bv. Va-1 LMG 19086	AJ270263	439	This study
<i>R. pickettii</i> bv. Va-1	X70348	294	Seal <i>et al.</i> (1993)
<i>R. pickettii</i>	AB004790	1468	–
<i>R. pickettii</i> bv. Va-2 LMG 5942 ^T	AJ270265	474	This study
<i>R. pickettii</i> bv. Va-2 LMG 5942 ^T	S55004	970	Yabuuchi <i>et al.</i> (1992)
<i>R. pickettii</i> bv. Va-2 ATCC 27512	X67042	1417	Li <i>et al.</i> (1993)
<i>R. pickettii</i> bv. Va-2 LMG 19085	AJ270266	450	This study
<i>R. pickettii</i> bv. Va-2	X70349	294	Seal <i>et al.</i> (1993)
<i>R. solanacearum</i>	X67036	1434	Li <i>et al.</i> (1993)
<i>Ralstonia</i> sp. LMG 19089	AJ270259	1455	This study, Suyama <i>et al.</i> (1998)
<i>Ralstonia</i> sp. LMG 19087	AB01438	447	This study, Suyama <i>et al.</i> (1998)
CDC group IVc-2 strain	AF098288	1495	Moissenet <i>et al.</i> (1999)
CDC group IVc-2 strain	AF067657	1527	Osterhout <i>et al.</i> (1998)
<i>Alcaligenes faecalis</i>	M22508	1475	Dewhirst <i>et al.</i> (1989)
<i>Brevundimonas diminuta</i>	M59064	1445	–
<i>Burkholderia andropogonis</i>	X67037	1435	Li <i>et al.</i> (1993)
<i>Burkholderia caryophylli</i>	X67039	1420	Li <i>et al.</i> (1993)
<i>Burkholderia cepacia</i>	M22518	1473	Dewhirst <i>et al.</i> (1989)
<i>Burkholderia gladioli</i>	X67038	1434	Li <i>et al.</i> (1993)
<i>Burkholderia mallei</i>	S55008	378	Yabuuchi <i>et al.</i> (1992)
<i>Burkholderia mallei</i>	S55000	962	Yabuuchi <i>et al.</i> (1992)
<i>Comamonas testosteroni</i>	M11224	1563	Yang <i>et al.</i> (1985)
<i>Pseudomonas aeruginosa</i>	X06684	1537	Toschka <i>et al.</i> (1988)

bv., Biovar.

phatase and phosphoamidase. Valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α - and β -galactosidase, β -glucuronidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase were not detected.

Cellular fatty acid composition

The cellular fatty acid composition was determined for *R. mannitolytica* strains LMG 6866^T, LMG 19090 and LMG 19091. Table 3 presents the data in comparison

with those obtained for other *Ralstonia* species. The principal components for the *R. mannitolytica* strains were 16:1^{9cis} (range 30–36 mol%) and 16:0 (range 21–30 mol%), with 7–15 mol% of 18:1^{11cis} and 5–7 mol% of 14:0.

DNA G + C content

The DNA G + C content for all three *R. mannitolytica* strains tested was 66.2 mol% (Table 4), which was higher than the values obtained for the four *R. pickettii*

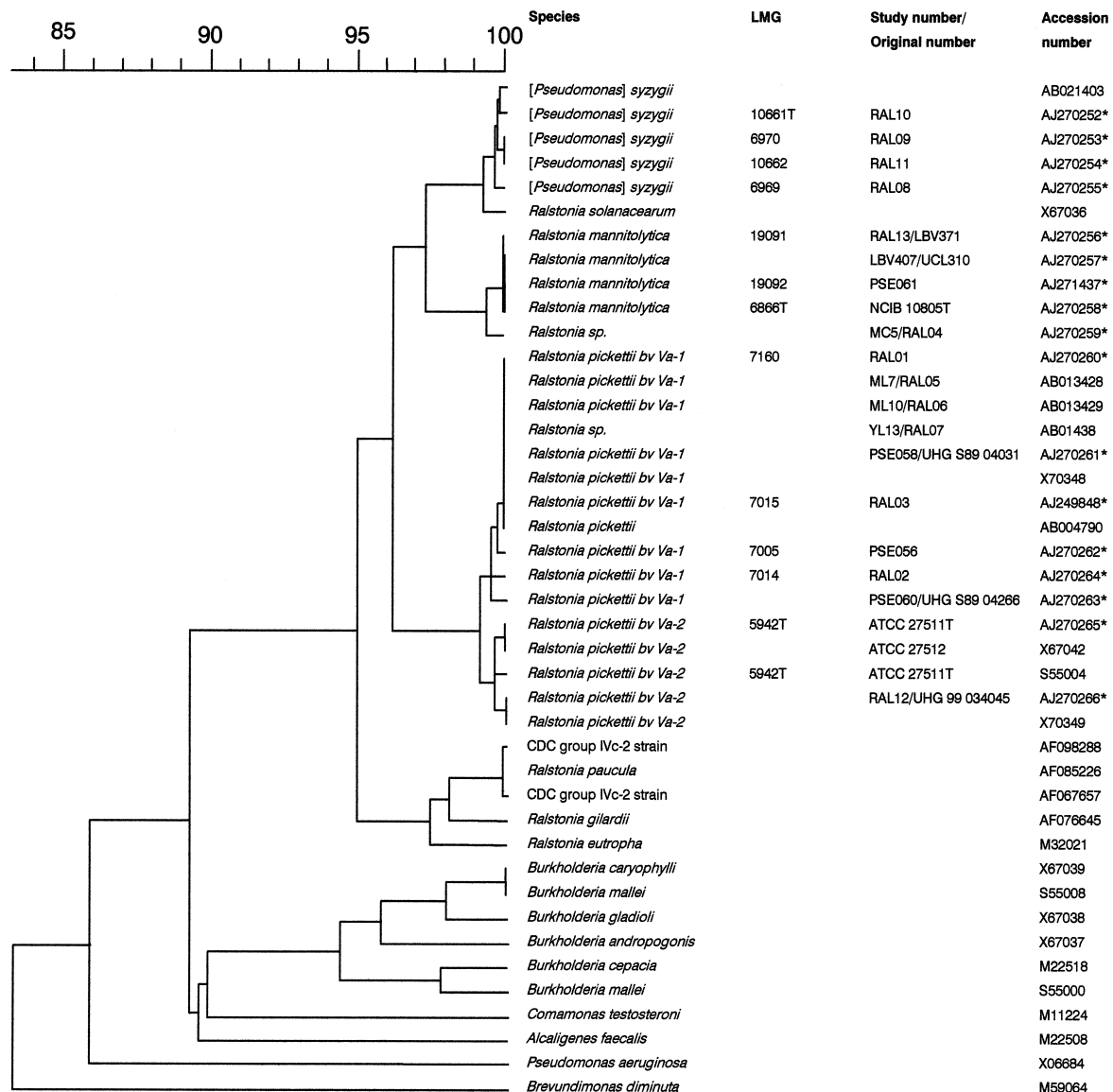


Fig. 1. Unweighted pair group method with averages (UPGMA) cluster analysis of the 16S rDNA sequences. Accession numbers indicated with asterisks refer to sequences determined in this study. The allocation of strains to either *R. pickettii* biovar Va-1 or Va-2 was done by testing acid production from maltose and lactose using OF medium: both positive for *R. pickettii* biovar Va-1 and both negative for *R. pickettii* biovar Va-2 (Pickett, 1994).

strains (64.0–64.1%) and for the two unnamed *Ralstonia* strains (64.0 and 65.1%).

16S rDNA sequence determination and total DNA homology

The 16S rDNA sequences included in the analysis are listed in Table 5. The 16S rDNA sequences of the three clinical strains LMG 19090, LMG 19091 and LMG 19092 were identical and clustered at more than 99.5% sequence similarity with the *R. mannitolytica* type strain, LMG 6866^T (Fig. 1). DNA–DNA hybridization confirmed that these three strains belonged to the same species (Table 4).

Closest to this group, with a 16S rDNA sequence similarity of more than 99%, was strain LMG 19089 (MC5), which was originally isolated from a brown lowland soil (pH 6.0) near the sandy bank of the Kinu river, Hama, Mitsukaido-Shi, Ibaraki Prefecture, Japan, and which was found to degrade poly(hexa-methylene carbonate) and poly(ϵ -caprolactone) and to accumulate poly(3-hydroxybutyrate) granules inside the cells (Suyama *et al.*, 1998). However, the level of DNA–DNA hybridization with three *R. mannitolytica* strains was only 56–58% (Table 4), so it can be concluded that this strain probably belongs to a separate, as-yet unclassified species.

The *R. solanacearum* 16S rDNA sequence (GenBank

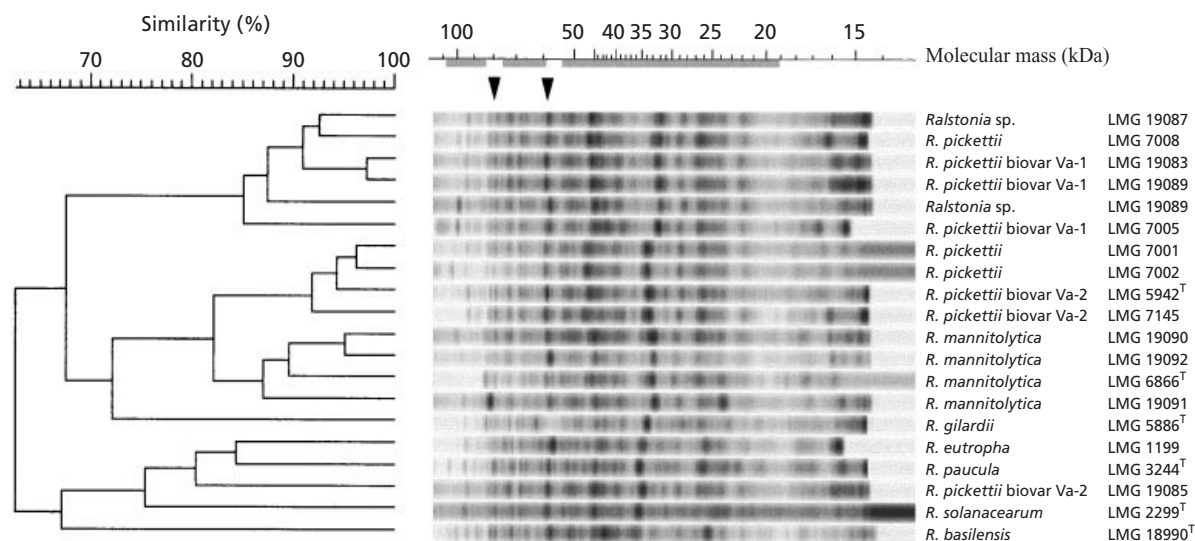


Fig. 2. Digitized representation of normalized SDS-PAGE protein profiles of different *Ralstonia* species and a dendrogram derived from the UPGMA linkage of correlation coefficients between the SDS-PAGE protein profiles of the strains studied. The zones indicated by arrowheads corresponding to molecular masses of approximately 60 and 80 kDa were omitted from the clustering.

accession no. X67036) clustered at more than 99% similarity with the sequences obtained for the [*P.*] *syzygii* strains LMG 6969, LMG 6970, LMG 10661^T and LMG 10662 (Fig. 1). LMG 19089 and the *R. mannitolytica* strains clustered with *R. solanacearum*/*[P.] syzygii* at less than 98% rDNA sequence similarity. The 16S rDNA sequences for the *R. pickettii* biovar Va-1 and Va-2 strains clustered at 96% similarity versus the LMG 19089–*R. mannitolytica*–*R. solanacearum*/*[P.] syzygii* cluster. The sequences of *R. paucula*, *R. gilardii* and *Ralstonia eutropha* strains apparently formed a separate cluster at a similarity of less than 96% to *R. pickettii*, *R. solanacearum*/*[P.] syzygii*, strain LMG 19089 and *R. mannitolytica*. The available 16S rDNA sequences of *Ralstonia* group CDC IVc-2 strains [GenBank accession nos AF098288 (Moissenet *et al.*, 1999) and AF067657 (Osterhout *et al.*, 1998)] were found to be 100% identical to that of a *R. paucula* strain (Vandamme *et al.*, 1999; GenBank accession no. AF085226). Sequence determination of the 5' end of the 16S rDNA revealed that the three *R. pickettii* strains found to produce no acid from maltose and lactose (i.e. *R. pickettii* biovar Va-2 strains) differed from the eight maltose and lactose acidification-positive *R. pickettii* strains (i.e. *R. pickettii* biovar Va-1 strains) at two positions. At *Escherichia coli* position 259, the A observed for *R. pickettii* biovar Va-2 is replaced by G for *R. pickettii* biovar Va-1. The other transition is at *E. coli* position 269, at which the T in the 16S rDNA sequence of *R. pickettii* biovar Va-2 is replaced by an A in *R. pickettii* biovar Va-1. The differences in biochemistry and 16S rDNA sequence are corroborated by the DNA–DNA hybridization data, since the two Japanese soil isolates (LMG 19083

and LMG 19088), which were identified as *R. pickettii* biovar Va-1 according to 16S rDNA sequence and biochemical characteristics, showed only 57–62% DNA relatedness to the two *R. pickettii* biovar Va-2 strains included in the DNA–DNA hybridization studies (Table 4).

PAGE of cell proteins

SDS-PAGE largely confirmed the 16S rDNA sequence and the DNA–DNA hybridization data. Strains LMG 6866^T, LMG 19090 and LMG 19091 belonged to a cluster with internal similarity of more than 80%, well separated from all other reference and non-reference strains. This grouping was only obtained when two small zones at 60 kDa (± 1 kDa) and 80 kDa (± 1 kDa), which contained a heavy protein band in the patterns of strains LMG 19090 and LMG 19091, were excluded from the similarity calculations (Fig. 2).

DISCUSSION

Clinical relevance of *R. mannitolytica*

A limited number of cases of hospital outbreaks with '*Pseudomonas thomasi*' and *R. pickettii* biovar 3/'*thomasi*' isolates have been reported in the literature (Baird *et al.*, 1976; Phillips *et al.*, 1972; Dowsett, 1972; Pan *et al.*, 1992). The first report (Phillips *et al.*, 1972) dealt with bacteraemia and bacteriuria in 25 patients due to the administration of parenteral fluids prepared at the hospital pharmacy, where deionized water contaminated with '*P. thomasi*' had been used (Phillips *et al.*, 1972; Phillips & Eykyn, 1972). Pan *et al.* (1992) reported that 23 of 39 *R. pickettii* isolates from

an epidemic involving 24 patients that was caused by contaminated saline solution (prepared by the hospital pharmacy) belonged to '*P. thomasii*'.

The clinical importance of *R. mannitolytica* may have been overlooked, possibly due to misidentification as *P. fluorescens*, which does not lead to further investigation since this organism is usually considered to be a contaminant. Indeed, after strain LMG 19090, initially identified as *P. fluorescens* (API profile code 0 054 555), had been recognized as *R. mannitolytica* because of colistin resistance and further biochemical testing, we studied retrospectively the only other strain (LMG 19091) in the GUH collection that had been stored as *P. fluorescens* (API profile code 0 045 555) and could identify it also as *R. mannitolytica*. Thus, microbiologists should consider the possibility of an identification as *R. mannitolytica* for colistin-resistant *P. fluorescens* isolates. Strain LMG 19092 belonged to a collection of strains that had been catalogued previously as belonging to an *R. pickettii* outbreak at the GUH in 1990 and thus adds to the number of nosocomial outbreaks caused by *R. mannitolytica*.

Differentiation of *R. mannitolytica* at the species and genus level

The distinctiveness of *R. mannitolytica* and *R. pickettii* biovar Va-1 and biovar Va-2 has been recognized previously. Although the study of King *et al.* (1979) was not conclusive and failed to establish '*P. thomasii*' as a valid species, Costas *et al.* (1990) concluded that SDS-PAGE of whole-cell proteins provided clear evidence that *R. pickettii* and '*P. thomasii*' were separate taxa, a finding confirmed by this study. On the other hand, Pan *et al.* (1992) reported that HPLC analysis of cell wall fatty acids could not distinguish *R. pickettii* biovar Va-1 from *R. pickettii* biovar 3/'*thomasii*', findings also confirmed by this study.

Several clear phenotypic differences exist between *R. mannitolytica* and the other *Ralstonia* species. *R. mannitolytica* can be differentiated from the other *Ralstonia* species, except for *Ralstonia* sp. strains LMG 19089 (RAL04/MC5) and LMG 19087 (RAL07/YL13), through its assimilation/acidification of mannitol and D-arabitol. *R. mannitolytica* strains differ from *R. pickettii* and *R. solanacearum* by their resistance towards desferrioxamine and from *R. pickettii* because of their lack of alkalization of tartrate and of nitrate reductase. The *R. pickettii* biovars Va-1 and Va-2 differ from each other by the lack of acid production from maltose and lactose for *R. pickettii* biovar Va-2 strains. The *R. solanacearum* strains tested could be differentiated from other species by acidification/assimilation of inositol and sucrose and lack of pyrrolidonyl arylamidase and of assimilation of caprate, malonate, propionate, suberate, acetate and lactate. *R. eutropha* strains were positive for alkaline phosphatase (Rosco) and assimilation of 3-hydroxybenzoate but did not alkalize mucate, in contrast to the other *Ralstonia* species. *R. paucula*

strains differed from *R. eutropha* and *R. gilardii* by strong urease production and from *R. eutropha* (no data available for *R. gilardii*) by alkalization of mucate but not of adipate and allantoin. Strains of *R. mannitolytica* were previously reported to be adonitol and ethanol acidification-negative, like the *R. pickettii* biovars Va-1 and Va-2, and cellobiose-positive, like *R. pickettii* biovar Va-1 (Pickett, 1994).

Yabuuchi *et al.* (1995) pointed to the difficulty of establishing reliable features to differentiate *Ralstonia* from *Burkholderia* at the generic level and stated that the three *Ralstonia* species (*R. pickettii*, *R. eutropha* and *R. solanacearum*) did not assimilate the carbohydrates galactose, mannitol, mannose or sorbitol, while the 11 *Burkholderia* species did. However, *R. mannitolytica* also assimilates and acidifies mannitol. As a consequence, differentiation between *R. mannitolytica* and *B. cepacia* genomovar II strains, which do not decarboxylate lysine or acidify sucrose (Vandamme *et al.*, 1997), is difficult, since *R. mannitolytica* and *B. cepacia* both produce acid from the same carbohydrates (with the exception of sucrose: 87% of *B. cepacia* strains are positive, whereas *R. mannitolytica* strains are negative) and since *R. mannitolytica* strains are unable to reduce nitrates, with *B. cepacia* strains unable to reduce nitrate to molecular nitrogen and with one-third of the *B. cepacia* strains reducing nitrate only to nitrite. Differentiation is only possible by means of a positive pyrrolidonyl arylamidase test for *R. mannitolytica*. Furthermore, in the routine clinical laboratory, *R. mannitolytica* can be differentiated from *P. fluorescens* and *Pseudomonas aeruginosa* by a negative pyoverdine test and from *P. fluorescens* by its inability to grow on *Salmonella/Shigella* agar and by a negative arginine dihydrolase test.

The Japanese environmental isolates LMG 19087 and LMG 19089 were phenotypically indistinguishable from each other, but according to 16S rDNA sequence and DNA-DNA hybridization studies they belong to two separate, as-yet undescribed *Ralstonia* species. LMG 19089 clusters most closely to *R. mannitolytica* according to its partially determined 16S rDNA sequence. In SDS-PAGE, it clusters close to *R. pickettii* biovar Va-1. Both strains can be differentiated from *R. mannitolytica* only by their ability to reduce nitrate and LMG 19087 also by lack of growth at 42 °C. LMG 19087 falls within the *R. pickettii* biovar Va-1 cluster according to its 16S rDNA sequence.

The Japanese environmental isolates LMG 19083 and LMG 19088 could be identified biochemically and according to 16S rDNA sequence and SDS-PAGE profile as genuine *R. pickettii* biovar Va-1 strains.

R. pickettii biovars Va-1 and Va-2 are easily distinguishable biochemically and some unambiguous 16S rDNA sequence differences are present, as shown in this study. DNA-DNA hybridization, as carried out here, separates the two representatives of each group tested, although Pickett & Greenwood (1980) found

84% DNA homology between a Va-1 strain and the type strain of *R. pickettii* (which is a Va-2 group strain) and Ralston *et al.* (1973) found 90% binding at 71 °C between Va-1 and Va-2 strains. In the SDS-PAGE clustering of whole-cell proteins, the *R. pickettii* strains seem somewhat heterogeneous, without clear-cut delineation between biovar Va-1 and biovar Va-2 strains.

Comparative 16S rDNA sequence analysis indicates that two sublineages may be discriminated within the genus *Ralstonia*, with the *R. eutropha* lineage on the one hand, which is composed of *R. eutropha*, *R. paucula* and *R. gilardii*, and the *R. pickettii* lineage on the other hand, with *R. pickettii*, *R. solanacearum*, *R. mannitolytica* and [*P.*] *syzygii*. This genotypic discrimination is supported by at least some phenotypic differences. Members of the *R. eutropha* lineage are colistin susceptible, do not produce acids from carbohydrates, assimilate oxalate but not galacturonate and have peritrichous flagella in contrast to members of the *R. pickettii* lineage, which are colistin resistant, have a polar flagellum and have biochemical characteristics distinct from those mentioned for the *R. eutropha* lineage. They also differ in viability on TSA at room temperature, with viability of at most 6 d for the *R. pickettii* lineage and at least 21 d for the *R. eutropha* lineage. Incomplete data are available for *R. gilardii*, which belongs to the *R. eutropha* lineage according to 16S rDNA sequence analysis, although it was reported to be monotrichous (Coenye *et al.*, 1999). Further studies, also including *R. gilardii* and *R. basiliensis*, will indicate whether these findings can be confirmed.

Description of *Ralstonia mannitolytica* sp. nov.

Ralstonia mannitolytica (man.ni.to'ly.ti.ca. N.L. adj. *mannitolytica* cleaving mannitol).

The species accommodates the *Ralstonia pickettii* biovar 3/'*thomasii*' strains and at least some of the strains known as '*Pseudomonas thomasii*'. Cells are Gram-negative, non-sporulating rods that are motile by means of one polar flagellum (motility was not observed for the culture collection *R. mannitolytica* type strain, LMG 6866^T). Aerobic growth is observed at 30, 37 and 42 °C and strains are viable for less than 6 d on TSA at room temperature. Catalase and oxidase tests are positive. Nitrate and nitrite are not reduced. Strains are resistant to desferrioxamine, O:129 and colistin. No acid is produced from ethylene glycol. Urease, pyrrolidonyl arylamidase (Rosco), Tween esterase and phenylalanine deaminase tests are positive. Indole and hydrogen sulfide production, alkaline phosphatase (Rosco), arginine dihydrolase, lysine and ornithine decarboxylases, aesculin and gelatin hydrolysis tests are negative. Acid is produced oxidatively from glucose, L-arabinose, lactose, maltose, mannitol, D-arabitol and D-xylose. Alkalinization occurs on minimal mineral agar with acetate, serine, malonate, β-alanine, 4-aminobutyrate, azelate, succinate, fumarate, butyrate, formate, malate, mucate, galacturonate, citrate, histidine and lactate, but not with

acetamide, adipate, alginate, allantoin, amygdalin, L-arginine, benzoate, L-ornithine, maleate or tartrate. Testing by means of API 20NE, API 50CH and ID 32GN indicates that the strains assimilate acetate, N-acetylglucosamine, L-alanine, L-arabinose, D-arabitol, 4-hydroxybenzoate, 3-hydroxybutyrate, caprate, citrate, fructose, galactose, gluconate, glucose, 2-ketogluconate, glycerol, histidine, D-lactate, malate, malonate, mannitol, L-proline, propionate, serine, suberate and D-xylose, but not adipate, amygdalin, L-fucose, glycogen, inositol, itaconate, 5-ketogluconate, maltose, mannose, melibiose, phenylacetate, rhamnose, ribose, sucrose, salicin, D-sorbitol or 3-hydroxybenzoate. Using the API ZYM system, the following enzymes are detected: C4 esterase, C8 esterase-lipase, C14 lipase, leucine arylamidase, acidic and alkaline phosphatase and phosphoamidase. Valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not detected. The following cellular fatty acid components are present: 16:1^{9cis} (range 30–38 mol%), 16:0 (21–30 mol%), 18:1^{11cis} (7–15 mol%) and 14:0 (5–7 mol%). The DNA G+C content is 66.2 mol%.

The type strain is LMG 6866^T (deposited as '*Pseudomonas thomasii*' in 1972) (= NCIB 10805^T) (Phillips *et al.*, 1972). The *R. mannitolytica* strains used in this study have been deposited in the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie Ghent, Belgium) as LMG 19090, LMG 19091 and LMG 19092.

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One Case Each of Recurrent Meningitis and Hemoperitoneum Infection with *Ralstonia mannitolilytica*

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Two clinical cases of infection with *Ralstonia mannitolilytica* are described: a recurrent meningitis on an implanted intraventricular catheter and an infected hemoperitoneum as a complication of a cholangiocarcinoma. The strains were first misidentified as *Pseudomonas fluorescens* and *Burkholderia cepacia*. Further testing lead to the identification as *Ralstonia pickettii* biovar 3/“*thomasii*,” which was recently shown to represent a separate species, *R. mannitolilytica* (List editor N. Weiss, *Int. J. Syst. Evol. Microbiol.* 51:795–796, 2001), originally described as *R. mannitolilytica* (De Baere et al., *Int. J. Syst. Evol. Microbiol.* 51:547–558, 2001). *R. mannitolilytica* can be distinguished from all described *Ralstonia* species by its acidification of D-arabitol and mannitol and by its lack of nitrate reduction and of alkalization of tartrate. In order to determine the true prevalence of infections with this species, colistin-resistant “*P. fluorescens*” strains and strains growing on *B. cepacia* selective medium deserve further attention.

CASE REPORTS

Case report 1. In 1997, a Caucasian woman, 38 years old, presented with fever of unknown origin. At the age of 17 she had received a ventriculoatrial draining for hydrocephalia after an intracerebral hematoma. Twenty years later, after a localized epileptic insult, further neurological testing and imaging pointed to a diagnosis of cavernous hemangiomas, for which she was treated surgically. The nonfunctional ventricular drain was partially removed, leaving the intrathoracic part in place. Postoperatively, the patient developed meningitis. Culture remained negative, and the patient was treated with 2 g of ceftriaxone intravenously (i.v.) twice daily during 2 weeks. Two days after antibiotic therapy was stopped, the meningitis recurred. Culture of the liquor yielded a gram-negative bacillus that could grow on *Burkholderia cepacia* selective medium (Mast Diagnostics, Merseyside, United Kingdom) and therefore was first identified as *B. cepacia*. The same organism was cultured from the removed intracerebral catheter segment. Ceftriaxone (2 g i.v. twice per day) was started, and ciprofloxacin (400 mg i.v. thrice per day) was added. Fever subsided and liquor cultures remained negative.

In February 1998, the patient presented in a private hospital with a generalized epileptic insult and high fever (39.5°C) and was treated with amoxicillin and clavulanic acid. The fever subsided, and the single blood culture, positive for “*Pseudomonas fluorescens*,” was considered contaminated. From March 1998 onwards, the patient had repeatedly febrile episodes and lost 10 kg of weight. Blood cultures were not performed. In November 1998, the patient was admitted in the same private hospital for high fever and tonic-clonic insults. Seven blood cultures were found positive for “*P. fluorescens*,”

and the patient was referred to the Ghent University Hospital for removal of the endovascular catheter segment. Cultures of the removed catheter were positive and were identified as *Ralstonia pickettii* biovar 3/“*thomasii*.” Retrospectively, it was shown that this was also the correct identification for the “*P. fluorescens*” isolates that had been obtained from the private hospital. The strain was resistant to ampicillin, gentamicin, temocillin, and aztreonam but was susceptible to cotrimoxazole, piperacilin, cefuroxime, cefotaxime, ceftazidime, imipenem, and quinolones. The patient was treated, according to the susceptibility testing results, with cotrimoxazole and doxycycline. Since then, the patient has been doing well.

Case report 2. In December 1997, primary cholangiocarcinoma with extensive hepatic involvement was diagnosed in a 32-year-old woman. Chemotherapy with *cis*-platinum and 5-fluorouracil was started with good clinical response and improvement of the hepatic lesions. Six months later, the patient was seen in the surgery department for partial resection of the liver in order to reduce the tumor mass and to improve the effect of chemotherapy. One week after the resection, computerized axial tomography (CAT) scanning revealed that the Kehr drain was leaking into the abdomen. A hemoperitoneum was diagnosed, and a review of the abdomen revealed bleeding of the right vena subhepatica. Intraoperatively, a specimen was taken from the hematoma for culture. After enrichment in thioglycolate broth, *Enterococcus* sp. and a gram-negative nonfermenting bacillus were isolated. Three days after review, the patient developed fever with peaks up to 39°C and antibiotic therapy with cefuroxime was started. Abdominal drainage fluid culture yielded the same gram-negative nonfermenter. This strain was resistant to ampicillin, gentamicin, colimycin, and temocillin but susceptible to cotrimoxazole, cefuroxime, and quinolones. Despite cefuroxime treatment, fever persisted and metronidazole was added. A CAT scan of the abdomen showed an excessive amount of free abdominal fluid. One week later, small numbers of the nonfermenter and of *Enterococcus*

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TABLE 1. Characteristics useful for differentiating *R. mannitolilytica* from other gram-negative nonfermenters^a

Phenotypic test used	Test results for:							
	<i>R. mannitolilytica</i>	<i>R. pickettii</i>	<i>R. solanacearum</i>	<i>P. aeruginosa</i>	<i>P. fluorescens</i>	<i>Pseudomonas putida</i>	<i>B. cepacia</i>	<i>B. multivorans</i>
Reduction of nitrate	–	+	+	+	–/+	–	V	+
Reduction of nitrite	–	+	–	+/-	–	–	–	–
Colistin susceptibility	R	R	R	S	S	S	R	R
Desferrioxamine susceptibility	R	S	S	R	R	R	R/S	S
Acidification of sucrose	–	–	+	–	V	–	+/-	–
Acidification of mannitol	+	–	–	V	V	V	+	+
Acidification of D-arabitol	+	–	–	–	ND	ND	ND	ND
Pyrrolidone peptidase	+	+	–	V	V	–	–	–
Trypsin ^b	–	–	–	+	+	+	–	–
Arginine dihydrolase	–	–	–	+	+	+	–	–
Lysine decarboxylase	–	–	–	–	–	–	+/-	V
Alkalinization of tartrate	–	+	+	ND	ND	ND	ND	ND
Pyoverdinin production	–	–	–	+/-	+	+	–	–
Growth on salmonella-shigella agar	–	–	–	+/-	+	+	–	ND

^a ND, not done; +/-, majority of strains positive; -/+, majority of strains negative; R, resistant; S, susceptible, and V, variable.

^b Trypsin = benzyl arginine arylamidase.

sp. could still be isolated from the abdominal drainage fluid. Antibiotherapy was switched to piperacillin and tazobactam, and a new abdominal drain was placed. As the patient remained febrile, drug fever was suspected and antibiotics were stopped. A subsequent fluid specimen again grew *Enterococcus* sp. and a gram-negative, nonfermenting bacillus, later identified as *R. mannitolilytica*. The patient's condition improved very slowly, and finally the patient was discharged from the hospital knowing that new intrahepatic lesions were detected on CAT scan.

Strain LMG 19090, obtained from patient 1, was isolated on conventional media and could grow on *Burkholderia cepacia* selective medium, containing 100 mg of ticarcillin/liter and 300 U of polymyxin B/ml. API 20NE (BioMérieux, Marcy l'Etoile, France) testing identified the strain as *P. fluorescens* (profile code 0 054 555). Because of colistin resistance, this strain was studied in more detail (3, 7, 13, 14), which led to an identification as *R. pickettii* biovar 3/"thomasii."

The further data gathered by means of polyphasic taxonomy led to the description of this biovar as a separate species, named *R. mannitolilytica*, referring to its characteristic acidification of mannitol, unlike all other described *Ralstonia* species (5). The original spelling of the specific epithet "*mannitolitytica*" (5) was corrected to "*mannitolilytica*" (8).

In retrospect, strain LMG 19091 from patient 2, which had been identified previously as *P. fluorescens* (API 20NE profile code 0 045 555), and strain LMG 6866^T, isolated at St. Thomas' Hospital (London, United Kingdom) during an outbreak and deposited as "*Pseudomonas thomasii*" in 1972 (NCIB 10805) (11, 12), could both be identified as *R. mannitolilytica*.

The G+C content for all three *R. mannitolilytica* strains tested was 66.2 mol%, which is higher than the values for *R. pickettii* (64.0 to 64.1%) (5). The 16S rDNA sequences of the clinical strains (GenBank accession numbers AJ270256 and AJ270257) were identical and clustered at more than 99.5% sequence similarity with the *R. mannitolilytica* type strain LMG

6866^T (GenBank accession number AJ270258) (5). The 16S rDNA sequences for the *R. pickettii* biovar Va-1 and Va-2 strains clustered at 96% similarity versus *R. mannitolilytica*. DNA hybridization confirmed that the two clinical strains and the type strain belonged to a separate species (5). When tRNA PCR was performed (1, 9), all three strains had a PCR fragment of 108.4 bp (standard deviation, 0.06 bp), in combination with one or two other variably present fragments. The obtained tRNA PCR fingerprints were sufficiently discriminative for us to recognize each strain as being *R. mannitolilytica*.

The two clinical *R. mannitolilytica* strains were motile by a single polar flagellum, while motility was not observed for the culture collection *R. mannitolilytica* type strain LMG 6866^T. It was observed that freshly isolated strains were very motile and that motility decreased upon prolonged preservation and subculture, which could explain the nonmotility of the type strain. All three strains grew at 30, 37, and 42°C and were viable for less than 6 days on tryptic soy agar (Becton Dickinson, Cockeysville, Md.) at 25°C. Oxidase and catalase were positive. They were resistant to desferrioxamine, O:129, and colistin. No acid was produced from ethylene glycol. Urease, pyrrolidonyl arylamidase (Rosco, Taastrup, Denmark), Tween esterase, and phenylalanine deaminase were positive. Acid was oxidatively produced from glucose, L-arabinose, lactose, maltose, mannitol, D-arabitol, and D-xylose. Alkalinization occurred on minimal mineral agar with acetate, serine, malonate, β-alanine, 4-aminobutyrate, azelate, succinate, fumarate, butyrate, formate, malate, mucate, galacturonate, citrate, histidine, and lactate but not with acetamide, adipate, alginate, allantoin, amygdalin, L-arginine, benzoate, L-ornithine, maleate, and tartrate (5).

In the routine clinical laboratory, *R. mannitolilytica* can be differentiated from *P. fluorescens* and *Pseudomonas aeruginosa* by a negative pyoverdinin test, by its inability to grow on salmonella-shigella agar, and by a negative arginine dihydrolase test (Table 1). Growth on *B. cepacia* selective medium pointed to an identification as *B. cepacia*. Differentiation from *B. cepacia*, especially from the genomovar II strains (i.e., *Burkholderia multivorans*), which do not decarboxylate lysine or acidify su-

crose (15), is difficult and is only possible by means of a positive pyrrolidonyl arylamidase test for *R. mannitolilytica*.

Finally, several clear phenotypic differences exist between *R. mannitolilytica* and the other *Ralstonia* species (Table 1). *R. mannitolilytica* can be differentiated from the other described *Ralstonia* species through its assimilation and acidification of mannitol and D-arabitol. *R. mannitolilytica* strains differ from *R. pickettii* and *Ralstonia solanacearum* by their resistance towards desferrioxamine and from *R. pickettii* because of their lack of alkalization of tartrate and of nitrate reductase. Strains of *R. mannitolilytica* were previously reported to be adonitol and ethanol acidification negative, like the *R. pickettii* biovars Va-1 and Va-2, and cellobiose positive, like *R. pickettii* biovar Va-1 (13).

A limited number of cases of hospital outbreaks with "*P. thomasii*" and *R. pickettii* biovar 3/"*thomasii*" isolates have been reported in the literature (2, 6, 10, 11). The first report (12) dealt with bacteremia and bacteriuria in 25 patients due to parenteral fluids prepared with deionized water contaminated with "*P. thomasii*" (11, 12). Pan et al. (10) reported that 23 of 39 *R. pickettii* isolates of an epidemic involving 24 patients that was caused by contaminated saline solution (prepared by the hospital pharmacy) belonged to "*P. thomasii*." A pseudo-outbreak has been described as well (4). Although no serious non-outbreak-related infections have been described thus far, the clinical importance of *R. mannitolilytica* may have been overlooked, possibly due to misidentification as *P. fluorescens*, *B. multivorans*, and/or *R. pickettii*.

We reported two cases of infection with *R. mannitolilytica*, first identified as *P. fluorescens* and/or *B. cepacia*. Colistin-resistant "*P. fluorescens*" isolates and strains growing on *B. cepacia* selective medium should be considered to be possibly *R. mannitolilytica*, a species that was formerly known as *R. pickettii* biovar 3/"*thomasii*" and that can be differentiated from *P. fluorescens* by its colistin resistance and its absence of arginine dihydrolase activity, from *B. cepacia* and *B. multivorans* by its pyrrolidonyl peptidase activity, and from other *Ralstonia* species by the acidification of mannitol. Correct identification of this organism may be of importance, since appropriate treatment was postponed in at least case 1, due to misidentification as *P. fluorescens* and *B. cepacia*, pointing to the presence of a

contaminant and also obscuring the long-term presence of the same bacterial organism.

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Case of Catheter Sepsis with *Ralstonia gilardii* in a Child with Acute Lymphoblastic Leukemia

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Acute lymphoblastic leukemia was diagnosed in a 7-year-old girl. Two months after insertion of a central venous catheter, she developed fever and complained of headache and abdominal pain. Physical examination revealed no focus of infection. A gram-negative nonfermenting bacillus was recurrently cultured from blood. Extensive biochemical testing and 16S ribosomal DNA sequencing led to the identification of *Ralstonia gilardii*.

CASE REPORT

Acute lymphoblastic leukemia (ALL) was diagnosed in a 7-year-old girl in May 2000, and treatment was initiated according to the EORTC-CLCG-58951 protocol for children with very-low-risk ALL. The girl achieved hematologic remission after induction chemotherapy. A central venous catheter was inserted in June 2000. The girl tolerated the treatment uneventfully until September 2000, when, during a course of chemotherapy (high-dose methotrexate), she developed spiking fever as high as 40°C. She complained of headache and abdominal pain and vomited twice. Physical examination revealed no focus of infection. The leucocyte count was 5,800/ml, with an absolute neutrophil count of 4,760/ml and elevated C-reactive protein (87 mg/dl). The chemotherapy was stopped, and the girl was treated with intravenous (i.v.) ampicillin (100 mg/kg of body weight/day). One day later blood cultures grew gram-negative bacilli, and (i.v.) netromycin (7.5 mg/kg/day) treatment was added. The spiking fever disappeared and the girl's health improved.

A gram-negative nonfermenting bacillus was isolated and found to be resistant to ampicillin, piperacillin, aztreonam, gentamicin, and tobramycin and susceptible to cefuroxime, ceftriaxone, ceftazidime, imipenem, co-trimoxazole, ofloxacin, and amikacin. The girl was treated as an outpatient with i.v. ceftriaxone (100 mg/kg/day) once daily for 4 more days. Thirty-six hours after the ceftriaxone treatment was stopped, she again developed spiking fever, and i.v. ceftriaxone (100 mg/kg/day) was restarted in combination with i.v. amikacin (15 mg/kg/day). Again, a gram-negative nonfermenting bacillus was cultured from blood. The girl showed an allergic reaction to ceftriaxone with rash and pruritus, and the ceftriaxone was replaced with i.v. ciprofloxacin (20 mg/kg/day). The spiking fever disappeared again, and amikacin and ciprofloxacin i.v. treatment was given for 7 more days. Blood cultures remained

negative, and the central venous catheter was not removed. Three months later the intensive chemotherapy was completed and the girl was doing well. The catheter was removed on January 8 2001. No nonfermenting gram-negative bacilli were cultured from the tip.

Discussion. The gram-negative bacillus was isolated in pure culture from all eight FAN aerobic blood cultures and from one of the eight BacT/Alert anaerobic cultures (Organon Teknika, Turnhout, Belgium), collected over a period of 10 days. Initial identification based on the API20NE system (bioMérieux, Marcy l'Etoile, France) yielded code 1000474, leading to an identification as *Alcaligenes faecalis*, *Comamonas acidovorans* or *Comamonas testosteronii*, or *Pseudomonas alcaligenes* or *Pseudomonas pseudoalcaligenes*. However, more elaborate biochemical testing led to the identification of the organism as *Ralstonia gilardii*. The strain in the present case report (designated GUH 00 09 2123); another clinical isolate of *R. gilardii* (UCL NF 926), isolated from a cerebrospinal fluid in 1977, without further information on clinical relevance; and three reference strains (LMG 5886^T, LMG 15537, and LMG 3400) were tested extensively. The bacteria were motile with peritrichous flagella, grew at 42°C, and were viable for more than 15 days on tryptic soy agar (TSA) at room temperature. Positive reactions were observed for catalase; oxidase; alkaline phosphatase (tablets; Rosco, Taastrup, Denmark); Simmons citrate; and alkalization of acetate, allantoin, lactate, and mucate on Simmons base agar. The strains were negative or very weakly positive for pyrrolidonyl arylamidase (Rosco) and gave a delayed positive result for alkalization of maleate on Simmons base agar. All five strains were susceptible to colistin and resistant to desferrioxamine. Negative reactions were observed for Tween 80 hydrolysis (read after 5 days); urease; phenylalanine deaminase; nitrite reduction; esculin and gelatin hydrolysis; arginine dihydrolase; ornithine decarboxylase; lysine decarboxylase; hydrogen disulfide (H₂S) and indole production; acidification of glucose, saccharose, maltose, manni-

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TABLE 1. Main differential phenotypic characteristics for *R. gilardii* and other oxidase-positive, motile, asaccharolytic nonfermenters^a

Characteristic	<i>Ralstonia gilardii</i>	<i>Ralstonia paucula</i>	<i>Bordetella bronchiseptica</i>	<i>Alcaligenes faecalis</i>	<i>Alcaligenes piechaudii</i>	<i>Achromobacter xylooxidans</i> subsp. <i>denitrificans</i>	<i>Comamonas testosteroni</i>	<i>Comamonas terrigena</i>	<i>Brevundimonas diminuta</i>
Flagellation	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Polar, tuft	Polar, tuft	Polar, single
Reduction of:									
Nitrate	V	–	+	–	+	+	+	+	–
Nitrite	–	–	–	+	–	+	–	–	–
Susceptibility to:									
Colistin (10- μ g disk)	S	S	S	S	S	S	S	S	R
Desferrioxamine	R	R	R	S	R	R	R	S	S
Production of:									
Pyrrolidone peptidase	–/+ ^w	+	–	–	+	+	+	–/+	–/+
Alkaline phosphatase (Rosco)	+/+ ^w	–	–	–	–	–	–	–	+
Urea hydrolysis	–	+	+	–	–	–	–	–	–

^a Symbols: +, positive; –, negative; –/+, majority of strains negative; +^w, weak reaction; –/+^w, negative or weakly positive. Abbreviations: V, variable; S, susceptible; R, resistant.

tol, arabinol, L-arabinose, inositol, lactose, and D-xylose; and alkalization on Simmons base agar of galacturonate.

Variable reactions were observed for alkalization of malonate, oxalate, and tartrate on Simmons base agar. Nitrate reduction tested by conventional methods was positive only for strains NF933 and LMG 3400, and it was additionally positive for strain LMG 15537, when tested with the API20NE system.

Reactions on ID32GN (BioMérieux) were positive for utilization of itaconate ($n = 5$), suberate ($n = 5$), acetate ($n = 5$), lactate ($n = 5$), L-alanine ($n = 4$), propionate ($n = 1$), caprate ($n = 5$), valerate ($n = 3$), citrate ($n = 1$), histidine ($n = 3$), 3-hydroxybutyrate ($n = 5$), 3-hydroxybenzoate ($n = 5$), and proline ($n = 5$). Reactions in the API20NE system (bioMérieux) were positive for gluconate ($n = 5$), caprate ($n = 5$), and malate ($n = 5$) and were variable for adipate ($n = 3$) and citrate ($n = 1$). This biochemical profile was consistent with an identification as *R. gilardii* (2, 3). It should be mentioned that all strains were found to have multiperitrichous flagella instead of a single polar flagellum as described previously (2). Table 1 summarizes the phenotypic characteristics used to differentiate *R. gilardii* from other oxidase-positive, motile, asaccharolytic, nonfermenting gram-negative rods.

Sequencing of 1,466 bp of the 16S rRNA gene was carried out as described previously (6) for the case report strain. The sequence obtained contained six ambiguities, which could not be resolved upon repeated sequencing and which are probably caused by the presence of multiple 16S rRNA operons with slightly differing sequences. Comparison to all known sequences of the GenBank by using the Blast program (<http://www.ncbi.nlm.nih.gov/blast>) resulted in a 98% similarity with two *Ralstonia* species strains (AF239160 and AY005039), and a *Ralstonia paucula* strain (AF067657). The only *R. gilardii* sequence present (LMG 5886^T [AF076645]) was only fourth in choice. This relatively low similarity could be largely explained by the fact that the sequence of the *R. gilardii* type strain (AF076645) contained 18 ambiguities. After detailed visual analysis of the sequences, only seven true mismatches were left, which raised the similarity to the highest observed, confirming the biochemical identification.

Using primers aimed at the amplification of tRNA intergenic spacer regions (1, 4, 5), no amplification signal could be

obtained, as is the case for most *Ralstonia* species (unpublished results). Therefore, tRNA-PCR appears not to be useful for the identification of most *Ralstonia* species.

R. gilardii may be of more clinical importance than is currently assumed but may have been largely overlooked due to identification problems and due to previously poor taxonomy. Indeed, the original publication describing this species (2) mentions several clinical strains that had been isolated from cerebrospinal fluid ($n = 2$), bone marrow ($n = 1$) and a furuncle ($n = 1$), without reference to published reports. A strain isolated from cerebrospinal fluid back in 1977 was present in our collection, and a new case of *R. gilardii* sepsis is reported here.

In summary, a gram-negative motile, nonfermenting, asaccharolytic bacillus with a positive oxidase and alkaline phosphatase reaction that is susceptible to colistin can be suspected to be *R. gilardii* and should warrant further identification, especially among the nonsaccharolytic nonfermenters.

Nucleotide sequence accession number. The sequence obtained for the present strain has been assigned GenBank accession no. AJ306571.

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VII. General discussion.

1. The introduction of DNA-fingerprinting techniques in a routine clinical microbiology laboratory.

Culture is - still – irreplaceable in the bacteriology and mycology laboratory because of its easiness for detection and because cultured microorganisms are still needed for antibiotic (antifungal) susceptibility testing (Vaneechoutte and Van Eldere, 1997).

This was the starting consideration to develop molecular techniques for the identification of cultured bacteria and fungi. Because applicability of these techniques in a routine clinical laboratory was an essential part of the aim, the PCR assays must be aimed at whatever organism present in the sample, but the number of primer sets should be kept minimal. This necessitates the use of broad-range or universal amplifications.

To identify organisms, variability between different species must be obtained, which can be done by fingerprinting PCR, resulting in fragment combinations obtained directly after PCR or after post-amplification manipulation.

Three different techniques were introduced or updated and evaluated.

The first technique used was ARDRA, which has the 16S rRNA gene as the universally present target (Wilson *et al.*, 1990) containing enough variability for discrimination between most bacterial species. Moreover, this gene is the basis for current phylogeny studies (Woese, 1987), so that the classification obtained by ARDRA could be correlated with the current taxonomy.

ARDRA was applied for the species identification of mycobacteria.

This technique was already in use in our laboratory (Vaneechoutte *et al.*, 1993), but the changes in culture devices required an update and re-evaluation of the technique. The liquid culture systems (Bactec (BD) and MB/BacT (Organon Teknika) are faster than solid culture (Löwenstein-Jensen) but contain more often contaminating growth, or contaminating bacterial DNA. Choosing a new primer pair more specific for the members of the genus *Mycobacterium* solved the problem of culture contamination with non-mycobacterial DNA. Furthermore, a simplified and more efficient DNA-extraction method was developed, the number of species was increased and a complete overview of all possible ARDRA patterns that can be encountered for *Mycobacterium* species was constructed, based on computer assisted restriction digestion of published 16S rRNA gene sequences.

A three-year evaluation indicated that all except three isolates could be correctly identified solely by this technique in an average time of 36 hours. The three isolates that could not be identified directly gave a fingerprint not present in the database. After identification of those isolates by other means (e.g. 16S rRNA sequencing), a species name could be linked to the unknown fingerprint, enabling identification of future isolates presenting this same fingerprint.

The experience with ARDRA for mycobacteria illustrates how the possibility to expand and adopt a library to new findings and/or taxonomic developments without redesigning the technology is a major advantage of DNA-fingerprinting techniques in general.

tRNA-PCR is a more broad-ranged PCR, proposed already 10 years ago as a universal fingerprinting PCR (Welsh and McClelland, 1991). The primers are a consensus, complementary to the edges of over 500 known tRNA gene sequences which are highly conserved in the bacterial world.

The major problem for using tRNA-PCR for routine identification is the interpretation of the obtained fingerprints. Analysis of the amplification products on a capillary electrophoresis system (Vanechoutte *et al.*, 1998) results in digital fingerprints, which are also very precise (up to one base pair differences can be detected). A software program (BaseHopper) especially designed for this purpose (Ir. Paul Baele, University Ghent), enables to correlate the fingerprint of an unknown isolate to a library of fingerprints obtained for reference strains. Digitalizing fingerprints results also in exchangeability of the obtained data, and evaluation of the interlaboratory reproducibility indicated that using the same capillary electrophoresis systems (ABI Prism 310) and analysis parameters, the data and libraries are exchangeable (Baele *et al.*, 2001).

The application is very successful for a majority of genera: *Staphylococcus* (Maes *et al.*, 1997); *Listeria* (Vanechoutte *et al.*, 1998); *Enterococcus* (Baele *et al.*, 2000); *Streptococcus* (Baele *et al.*, 2001) and *Lactobacillus* (Baele *et al.*, 2002). However for certain genera (e.g. *Ralstonia*, *Corynebacterium*) the fingerprints consist sometimes of only one or a few bands.

Our major attempt was to construct a library containing all possible clinical species. Unpublished preliminary data show that identification of routine clinical isolates is promising.

For a total of 313 isolates, identification both with the routine biochemical testing and with tRNA-PCR was carried out. 224 identifications (71.6%) were found in agreement. For 19 isolates (6%) the routine methods were found to be correct or more discriminatory, and in 70 cases (22%) tRNA-PCR was better than routine methods with regard to correctness and discriminatory power, e.g. because it could identify *Enterococcus*, *Staphylococcus* and *Streptococcus* sp. and non-fermenting Gram negatives rapidly to the species level (unpublished data).

Current problems of identification with tRNA-PCR are frequently caused by insufficiencies of the library (missing the species fingerprint of the unknown) or by taxonomical vagueness.

Besides the bacteria also yeast and molds are clinical isolates, which can be identified by DNA-fingerprinting techniques. The technique published by Turenne (Turenne *et al.*, 1999) was adapted to our current settings. We expanded the library of Turenne, adapted it to the usage of dTTP in stead of dUTP, and especially we developed a simple and very fast boiling/freezing DNA-extraction, and showed that it can replace the very laborious and time-consuming DNA-extraction methods generally used for yeasts.

Labeling the ITS2-PCR forward primer with another fluorescent dye than the tRNA-PCR forward primer enabled us to analyze fingerprints of a bacterial species and a fungal species at the same time. The current setting can maximally identify 36 bacterial and 36 fungal isolates during one day with still one fluorescent dye not used; so another 36 organism can be identified. The capacity of multiple capillary devices (e.g. ABI Prism 3100 (Applied Biosystems), SEQ 8000 (Beckman Coulter)) already exceeds that needed in a routine laboratory.

2. The use of the DNA-based identification techniques, leading to newly or infrequently encountered species.

The developed DNA-fingerprinting techniques result mostly in rapid and precise identifications, and using these techniques on large numbers of isolates leads sometimes to unknown fingerprints. Further investigation can indicate that either the species was not present in the library (with the tRNA-PCR library containing at present approximately 500 species and the ITS2-library 26 clinical important yeast species), or that we deal with a previously unknown species.

Use of ARDRA on a large collection of clinical isolates belonging to the genus *Acinetobacter*, made it possible to delineate certain groups of strains presenting a unique fingerprint not yet seen. Extensive biochemical analysis of those strains gave characteristics indicating the presence of a new species. For further confirmation a polyphasic approach was followed using for example 16S rRNA gene sequencing and DNA-DNA hybridizations, leading to the description of new species (Vaneechoutte *et al.*, 1999; Nemeč *et al.*, 2001).

An important advantage of a digitized technique like tRNA-PCR is that it easily provides an overview of the isolated species, such that frequently encountered organism can be noticed. The technician using only morphological and biochemical tests will not further identify clinically infrequent isolates, or has not the test to do so and thus will miss a final identification. The use of ARDRA led to a case report on *M. interjectum*, ITS2 revealed the presence of formerly not encountered *Dekkera bruxellensis*, and the use of tRNA allowed the description of several species, for example: *Enterococcus cecorum* (De Baere *et al.*, 2000), *Moraxella atlantae* (De Baere *et al.*, 2002) and *Leclercia adecarboxylata* (De Baere *et al.*, 2001).

3. The advantages and disadvantages for clinical applications.

Introduction

We have tried to show how culture will remain an indispensable tool in the clinical bacteriology laboratory, also in the distant future, if only because it will remain impossible to cover all the different resistance mechanisms in a genotypic manner. As such, primary identification using selective and non-selective media will remain the start of routine bacteriological examinations. The experience of a clinical microbiologist in deciding which colonies should be studied further will remain indispensable, and the primary culture medium will continue to supply with a semi-quantitative measure of the different organisms present, something which will remain difficult with genotypic approaches, even with real time PCR. As mentioned above, also susceptibility testing will remain based on culture, if only for practical reasons. One can then ask whether it might be useful to replace current phenotypic identification of the different colonies by a genotypic approach, as has been pursued for over a decade at our research laboratory. To answer that question, one should carefully analyse the different parameters used to evaluate the usefulness of routinely applicable techniques.

Evaluation of different parameters to compare the routine applicability of phenotypic and genotypic identification:

- **Speed.** Phenotypic identification of organisms like *E. coli* and *P. aeruginosa* is obtained within hours, while it can take days to weeks for anaerobic bacteria and nonfermenters, and often no identification can be obtained. Genotypic identification using tRNA-PCR takes 6 hours to one day for whatever organisms is studied.
- **Cost.** Calculations have shown that the cost of tRNA-PCR and ITS2-PCR is about 2-3 Euro, a price which is certainly competitive with phenotypic identification.
- **Discriminatory power.** tRNA-PCR has definitely a higher discriminatory power for *Enterococcus*, *Staphylococcus*, *Streptococcus* and *Lactobacillus* and performs much better for Gram-negative nonfermenting bacteria. Problems are encountered for the identification of *Corynebacterium* and *Mycobacterium*, and this might reflect the phylogenetic distance of this group of organisms. It should be noticed that ARDRA performs perfectly for *Mycobacterium* and *Corynebacterium*, and it is no major disadvantage to need another simple genotypic technique for mycobacteria, since the phenotypic identification alternatives are more cumbersome and are only carried out in reference laboratories, and other genotypic alternatives are usually more laborious and expensive. Another possibility to solve this problem is the design of more universal tRNA-primers. The discriminatory power for *Moraxella* species is excellent, it is moderate for *Acinetobacter* and *Pasteurellaceae* and poor for *Neisseria*. Another problem is the impossibility for reliable differentiation between *S. pneumoniae* and the other *S. mitis* group species, although the problem is largely solved by the fact that *S. pneumoniae* is already identified on the primary isolation plate by its optochin susceptibility. In general, a preliminary study of 500 routine isolates, identified with both phenotypic techniques and tRNA-PCR showed that the discriminatory power of the latter was higher. The discriminatory power of ITS2-PCR is higher than that of phenotypic identification.
- **Intraspecies variability.** Using phenotypic techniques, one is often confronted with variability within a species. Apparently, this is also the case for tRNA-PCR. ITS2-PCR shows less intraspecific variability. This has forced us to take up sometimes several fingerprints for one species into the library. This might be a problem by blurring the boundaries between species, but on the other hand this 'intraspecific variability' also sometimes has been indicative for the presence of separate species

within one ‘species’. This was e.g. the case for *Comamonas terrigena* (Wauters *et al.*, in press) and *Enterobacter cloacae* (in preparation).

- **Possibilities for automatisation.** These genotypic identification techniques are automatizable to a large extent and one technician could identify up to 50 strains per day, with only 50% hands on time.
- **Technical simplicity.** An advantage of the genotypic approaches is that, whatever species is studied, the handling remains the same (briefly: DNA-extraction, PCR, electrophoresis, software based comparison of the obtained fingerprints with the library), and the manipulations can be learned within a week. To the contrary, phenotypic identification requires serious technical expertise, many different reagents, and many different decision trees, depending upon the organism one is dealing with. It should be noticed that, also with regard to quality control of the laboratory, one can envisage that it is much simpler to establish QC for a limited number of genotypic techniques (without the use of living material), than for a whole array of biological assays, requiring also standard organisms and standard culture conditions for these.
- **Interlaboratory reproducibility.** During this study it was sufficiently shown that the tRNA and ITS2-fingerprints obtained in one laboratory can be compared with those obtained in another laboratory (Baele *et al.*, 2001, De Baere *et al.* 2002). At several further occasions it was possible to identify strains for which a fingerprint was obtained in one laboratory, by using a library composed of fingerprints obtained at another laboratory. Also see ‘Future prospects’ on how to increase reproducibility. Interlaboratory reproducibility of phenotypic techniques might be estimated to be high, although problems may occur since each laboratory uses its own variations and combinations of phenotypic techniques.
- **Reliability.** The obtained identifications should be reliable, i.e. no misidentifications should be obtained, and in case an organism has not yet been encountered previously, no identification should be obtained. Using tRNA-PCR, the latter was the case for several species which we observed for the first time in our laboratory (e.g. *Moraxella atlantae*, *Leclercia adecarboxylata*). In a preliminary comparative study (using 500 routine strains), the reliability of tRNA-PCR was found to be higher than that of phenotypic identification. For example, on several occasions, strains were identified as *K. pneumoniae* in the routine laboratory, while tRNA-PCR yielded *E. aerogenes* as an identification, later confirmed by 16S rDNA sequence determination. ITS2-PCR, for

which identification is based on the length of only one fragment is more prone to misidentification, E.g. *Pichia anomala*, has a ITS2-fragment size that differs less than 1bp difference from *Cryptococcus neoformans*.

- **Flexibility.** When previously not encountered species have been studied, and a final identification has obtained, e.g. by means of 16S rDNA sequence determination, it is possible to simply add the new fingerprint to the library, such that further isolates will be identifiable.
- **Need for specialized equipment.** The DNA-fingerprinting techniques used in this study rely on precise size determination. This can only be obtained with specialized electrophoresis equipment. This might be a drawback, although one should keep in mind that current phenotypic identification machines cost more and moreover that the fluorescent electrophoresis equipment used here (e.g. ABI310) can also be applied for typing bacteria, for sequence determination and for other, non microbiological, fingerprinting techniques (e.g. as used in medical genetics).

4. Future prospects.

It is possible to develop tRNA-PCR and ITS-PCR as practically applicable techniques, aimed at identification of cultured bacteria and fungi, in the clinical laboratory. For example, one could devise commercially prepared, ready made microplates containing PCR mixture and the appropriate primers, such that, starting from a single colony and after a brief and simple DNA-extraction, an amplification reaction can be performed. This approach would not only simplify the handling and shorten the hands on time, but would also increase the interlaboratory reproducibility. Transfer of the amplification products to another microtiterplate, containing the size markers in the DNA-denaturing formamide mixture, is than a matter of minutes with a minimum of hands on time. Using multiple capillary electrophoresis for the size determination of the amplified DNA-fragments (i.e. intergenic tRNA spacers for the bacteria or ITS2 fragments for the yeasts) one can obtain tens of fingerprints within a few hours after picking the individual colonies. Final identification of the obtained fingerprints with an already existing, and continuously refined and expanding, library, can be performed in an automated fashion as well. The remainder of the colony used for genotypic identification, can be used for susceptibility testing. We think, given the results obtained in this study, that this approach certainly might compete with current phenotypic identification with regard to cost, speed and discriminatory capacity. Of course, phenotypic

identification is making continuously progress, and fully automated identification machines (like Vitek or Phoenix) are on the market, although currently fifty percent of the cultures need an extra overnight incubation in order to obtain sufficient inoculum for a reliable identification. The future will show whether one of these or still other techniques will be used.

VIII. Summary

Since the description of the polymerase chain reaction and the introduction of a thermostable polymerase, some 15 years ago, most research in bacterial and mycological diagnostics has been invested in the application of detection of these organisms, directly from clinical samples. The fact that such applications have been applied and commercially developed only for a few slow growing organisms, like *Mycobacterium tuberculosis*, indicates that broad-range application of PCR-based detection struggles with several problems in bacteriology and mycology. Not only are bacterial and fungal culture techniques reasonably fast and easy and do they provide with semi-quantitative information on a vast array of different organisms, culture is also needed for susceptibility testing of the detected organisms, since it remains impossible to cover all the resistance genes with DNA-techniques in a practical manner, despite the development of DNA-arrays. Thus, even if DNA-based detection was possible, it would not circumvent culture, which still would be necessary for susceptibility testing.

Although culture for detection cannot be circumvented, one can wonder whether phenotypic identification can be replaced by PCR-based DNA-fingerprinting techniques aimed at identification of cultured micro-organisms. Indeed, phenotypic identification has several shortcomings, such as the requirement for a combination of different approaches (serology, morphology, biochemistry) and different tests, the sometimes limited discriminatory power and considerable experience.

In this doctoral work we updated and introduced PCR-based DNA-fingerprinting for mycobacteria (ARDRA), bacteria in general (tRNA-PCR) and yeasts (ITS2-PCR). The number of species identifiable with these techniques was expanded, the techniques were standardized and were applied to the identification of clinical bacterial and fungal isolates. Whenever discrepancies were observed between routine phenotypic identification and the molecular identification, DNA-sequencing was carried out of the 16S rRNA-gene or the ITS2-region. This led to the continuous expansion and refinement of the fingerprint libraries and in some cases this resulted in the description of new species of highly infrequently encountered species.

VIII. Samenvatting

Sinds de publicatie van de polymerase ketting reactie en de introductie van een thermostabiel polymerase, zo'n 15 jaar geleden, werd het merendeel van het onderzoek in bacteriële en mycologische diagnostiek gericht naar de detectie van deze organismen, rechtstreeks uit het staal. Het feit dat deze toepassingen, net als de commercieel ontwikkelde testen, enkel gericht zijn op een klein aantal traag groeiende organismen, zoals *Mycobacterium tuberculosis*, geeft aan dat meer algemeen gericht toepassingen van PCR-gebaseerde detectie te kampen hebben met ernstige problemen in de bacteriologie en mycologie.

Naast het feit dat bacteriële en mycologische kweek relatief eenvoudig en snel is, is er ook nog de bijkomend semi-kwantitatieve informatie bekomen voor een zeer uiteenlopende verscheidenheid van organismen. Tevens is kweek nog steeds noodzakelijk voor de antibiotica (of antifungica) gevoeligheidsbepalingen van gedetecteerde organismen, daar het nog steeds onmogelijk lijkt alle mogelijke resistentie genen te controleren met praktisch haalbare DNA-technieken, zelfs ondanks de ontwikkeling van DNA-arrays.

Niettegenstaande echter dat kweek onvervangbaar blijkt te zijn voor detectie, kan men zich afvragen of het niet mogelijk zou zijn de fenotypische identificatie te vervangen door PCR-gebaseerde DNA-fingerprinting technieken voor de identificatie van gekweekte organismen. Er zijn inderdaad tekortkomingen aan de fenotypische identificatie zoals de behoefte aan een combinatie van verschillende benaderingen (serologie, morfologie en biochemie) en testen, alsook het soms beperkt discriminerend vermogen.

In dit doctoraatswerk werden PCR-gebaseerde DNA-fingerprinting technieken ofwel herzien ofwel geïntroduceerd voor de identificatie van mycobacteriën (ARDRA), bacteriën in het algemeen (tRNA-PCR) en gisten (ITS2-PCR).

The aantal species identificeerbaar met deze technieken werd uitgebreid, de technieken werden gestandaardiseerd en toegepast voor de identificatie van klinisch isolaten (bacteriën en gisten). Wanneer er geen overeenstemming was tussen de routinematig gebruikte fenotypische identificatie en de moleculaire identificatie werd ofwel 16S rRNA of gen ofwel ITS2 sequentiebepaling uitgevoerd, teneinde de juiste identificatie te bekomen, welke dan weer leidde tot de continue uitbreiding en verfijning van de fingerprint bibliotheek. Tevens leidde dit in sommige gevallen tot het opmerken en beschrijven van nieuwe of heel uitzonderlijk voorkomende species.

VIII. Résumé

Depuis la publication de la réaction en chaîne de polymérase (PCR) et l'introduction d'une polymérase thermostable, il y a 15 ans environ, la plus grande partie de la recherche dans le domaine du diagnostic bactérien et mycologique a été orientée vers la détection de ces organismes sans passer par l'étape de culture. Le fait que ces techniques ne soient développées commercialement que pour un nombre limité d'organismes à croissance lente comme *Mycobacterium tuberculosis*, indique qu'une application plus généralisée de la détection basée sur la PCR rencontre plusieurs problèmes.

Les techniques de culture bactérienne et mycologique sont non seulement raisonnablement rapides et faciles et nous fournissent une information semi-quantitative concernant une grande série d'organismes, la culture est nécessaire aussi pour tester la sensibilité (antibiotique (ou antifongique)) des organismes détectés. En effet il est toujours impossible pratiquement de contrôler tous les gènes de résistance avec des techniques PCR, malgré le développement de 'arrays d'ADN'.

Dès lors, même si la détection basée sur ADN était possible, la culture resterait indispensable pour tester la sensibilité.

Bien que la culture ne puisse pas être éliminée pour la détection, on peut cependant se demander si l'identification phénotypique ne pourrait pas être remplacée par des techniques d'empreintes génétiques par PCR, afin d'identifier les micro-organismes cultivés.

L'identification phénotypique présente effectivement plusieurs imperfections comme le besoin d'une combinaison d'approches (sérologie, morphologie, biochimie) et de tests différents, et qui sont de pouvoir discriminatoire souvent limité.

Dans ce doctorat nous avons adapté et introduit des techniques d'empreinte génétique ADN basées sur la technique de PCR pour l'identification de mycobactéries (ARDRA), bactéries en général (tRNA-PCR) et de levures (ITS2-PCR).

Le nombre d'espèces identifiables par ces techniques a été augmenté, les techniques ont été standardisées et appliquées à l'identification d'isolats cliniques bactériens et mycologiques.

Dans le cas de non concordance entre l'identification phénotypique routinière et l'identification moléculaire, la détermination de séquences a été faite sur les gènes 16S rRNA ou ITS-2. Cela a contribué à la création d'une banque de données d'empreintes génétique constamment complétée et raffinée. En plus dans quelques cas, on a pu réaliser la description d'espèces nouvelles ou très rares.

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Thierry.

XI. Curriculum

Thierry De Baere werd geboren op 6 mei 1972 te Deinze. Na het beëindigen van het secundair onderwijs, richting Wetenschappen B, in het Heilig-Hart college te Waregem, startte hij in 1990 met de studies Scheikunde aan de Universiteit Gent, en in 1992 aan de Licenties in de Biotechnologie, welke hij in 1994 met onderscheiding voltooide.

Na opdrachten bij Industrial Plastics en Omega Pharma, kwam hij in 1996 terug naar het wetenschappelijk onderzoek op het Universitair Ziekenhuis Gent, in een IWT project samen met Innogenetics en het Instituut voor Tropische Geneeskunde. Wanneer dit in 1998 ten einde liep kon hij verder werken als wetenschapper binnen een Geconcentreerde Onderzoeksactie (GOA) met de departementen microbiologie van de Faculteit Diergeneeskunde en de Faculteit Wetenschappen. In 2000 kreeg hij een assistentschap voor de periode van 4 jaar binnen de vakgroep Klinische Biologie, Microbiologie en Immunologie.

In 1998 slaagde hij voor de post-academisch opleiding: Medische Mycologie; en in 2002 voltooide hij de interuniversitaire doctoraatsopleiding.

Thierry De Baere is auteur of co-auteur van 27 publicaties in internationale tijdschriften en was spreker op een internationaal congres. In 1997 behaalde hij een Glaxo-Wellcome grant for young scientist.

Thierry heeft samen met Véronique Colpaert twee kinderen: Camille en Maxime.

Publicaties in internationale tijdschriften:

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- PCR-based identification of cultured mycobacteria in routine diagnostic laboratory. 1999. International Colloquium : Tuberculosis, the real millenium bug. December 14-16. Antwerp, Belgium.
- Rapid and reliable identification of the clinically important fungal species by automated capillary electrophoresis of the amplified ITS2 spacer region. 2001. Belgische vereniging voor Menselijke en Dierlijke mycologie - jaarvergadering symposium: De mycologische diagnose. Februari 3, Wilrijk, Belgium.