

Identification of staphylococci by 16S internal transcribed spacer rRNA gene restriction fragment length polymorphism

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The capacity of 16S internal transcribed spacer (16S-ITS) rRNA gene RFLP to differentiate 16 type strains and nine clinical isolates of staphylococci was evaluated. The 16S rRNA gene was amplified together with the ITS region and the amplification products were digested with *TaqI* restriction enzyme. Analysis of the 16S-ITS rRNA gene RFLP profiles differentiated each of the 16 type strains into distinct RFLP haplotypes.

INTRODUCTION

The habitat of staphylococci is skin, skin glands and mucous membranes of humans and many animals. The genus *Staphylococcus* includes both pathogenic and saprophytic strains that have been isolated from animal products such as meat and milk, and from environmental sources such as soil, sea water, fresh water, dust and air samples (Kloos *et al.*, 1991).

Coagulase-positive species such as *Staphylococcus aureus* are the cause of many types of infection (Forbes *et al.*, 1998). During the last two decades, coagulase-negative staphylococci (CNS) have also emerged as pathogens causing medical-device-related infections (von Eiff *et al.*, 2002). Because of the pathogenic potential of these bacteria, effective methods are required for their *in vitro* identification. Beside fatty acid analyses, many diagnostic tests rely on a miniaturized phenotypic characterization and a set of biochemical reactions. These methods enable the identification of *S. aureus* isolates, but often fail in the identification of CNS (Stoakes *et al.*, 1994; Renneberg *et al.*, 1995; Martineau *et al.*, 1996). Analysis of specific regions of genomic DNA, on the other hand, has produced much more discriminative data. For example, several genomic targets have been effectively used for the identification of *Staphylococcus* species, including the 16S rRNA gene (Bialkowska-Hobrzanska *et al.*, 1990; De Buyser *et al.*, 1992), the tRNA gene intergenic spacer (Maes *et al.*, 1997), the internal transcribed spacer (Couto *et al.*, 2001), the heat-shock protein 60 (*HSP60*) gene (Goh *et al.*, 1996), the chaperonin 60 gene (Goh *et al.*, 1997), the *femA* gene (Vannuffel *et al.*, 1999), the *sodA* gene (Poyart *et al.*, 2001), the *gap* gene (Yugueros *et al.*, 2000) and the *nuc* gene (Brakstad *et al.*, 1992). Recently, an enterobacterial repetitive intergenic consensus PCR and

BOX-PCR were also used in the identification of *Staphylococcus epidermidis* strains (Wieser & Busse, 2000).

The 16S internal transcribed spacer (16S-ITS) rRNA gene RFLP method has already been used for the identification of bacteria of diverse origin: lactic acid bacteria (Bulut *et al.*, 2005), lactobacilli (Yavuz *et al.*, 2004a), thermophilic bacilli (Yavuz *et al.*, 2004b) and alkalophilic bacilli (Akbalik *et al.*, 2004). In the present work, this method was used for the species-level identification of 16 staphylococcal type strains and nine clinical isolates that had been isolated from human and animals.

METHODS

Bacterial strains. The type strains used in this study were *S. aureus* (ATCC 25923^T), *Staphylococcus arlettae* (NRRL B-14764^T), *Staphylococcus auricularis* (NRRL B-14762^T), *Staphylococcus capitis* subsp. *capitis* (NRRL B-14752^T), *Staphylococcus caprae* (NRRL B-14757^T), *Staphylococcus carnosus* (NRRL B-14760^T), *Staphylococcus chromogenes* (NRRL B-14759^T), *Staphylococcus cohnii* subsp. *cohnii* (NRRL B-14756^T), *Staphylococcus condimentii* (DSM 11674^T), *Staphylococcus epidermidis* (NRRL B-4268^T), *Staphylococcus equorum* (NRRL B-14765^T), *Staphylococcus gallinarum* (NRRL B-14763^T), *Staphylococcus intermedius* (CCM 5739^T), *Staphylococcus lugdunensis* (NRRL B-14774^T), *Staphylococcus simulans* (NRRL B-14753^T) and *Staphylococcus xylosus* (DSM 20266^T). The nine clinical isolates were *S. capitis* subsp. *capitis* ($n = 2$), *S. caprae* ($n = 2$), *S. cohnii* subsp. *cohnii* ($n = 1$) and *S. epidermidis* ($n = 4$). The clinical isolates were identified by basic phenotypic tests and the Staph ID 32 system (bioMérieux). All strains were cultured in tryptic soy broth (TSB; Merck) at 37 °C and stored in 25 % (v/v) glycerol at –80 °C.

16S-ITS rRNA gene PCR. Genomic DNA was isolated as described by Arciola *et al.* (2001). Briefly, 100 µl of overnight culture prepared in 5 ml TSB at 37 °C with shaking was pelleted by centrifugation. Cell pellets were resuspended in 45 µl deionized water and 5 µl lysostaphin solution (100 µg ml⁻¹ in dH₂O; Sigma) and incubated for 10 min at 37 °C. Five microlitres of proteinase K solution (100 µg ml⁻¹ in dH₂O; Merck) and 150 µl 0.1 M Tris/HCl (pH 7.5) were added and the cells were further incubated for 10 min at 37 °C. The samples were heated for 5 min at

Abbreviations: CNS, coagulase-negative staphylococci; ITS, internal transcribed spacer.

100 °C and stored at -20 °C. For *S. caprae* and *S. aureus* strains, 15 µl lysostaphin, 15 µl proteinase K solution and a longer incubation time (1 h at 37 °C) were used. The 16S-ITS region was amplified using the primers 5'-AGAGTTTGATCCTGGCTCAG-3' (Mora *et al.*, 1998) and 5'-CAAGGCATCCACCGT-3' (Jensen *et al.*, 1993). The 50 µl reaction volume contained 1.2 U *Taq* polymerase (MBI Fermentas), 5 µl 10× reaction buffer [100 mM Tris/HCl pH 8.8, 500 mM KCl, 0.8% (v/v) NP-40 and 1.5 mM MgCl₂], 10 pmol of each of the primers (MWG Biotech), 0.2 mM of each of the four dNTPs (MBI Fermentas) and 5 µl of the bacterial lysate as the DNA template. PCR amplification was performed in a Techne Progene thermal cycler using the following amplification conditions: 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 42 °C and 1 min at 72 °C. Reactions were terminated with a 10 min final extension step at 72 °C. Amplification products were analysed by 0.8% (w/v) agarose gel electrophoresis.

RFLP. The amplified 16S-ITS rRNA gene fragments (~200 ng) were digested with 5 U *TaqI* (MBI Fermentas) restriction enzyme for 3 h at 65 °C. Restriction products were resolved on a 2% (w/v) agarose gel (low EEO; Applichem) for 1 h at 40 mA and 3 h at 60 mA in 1× TAE (0.04 M Tris-acetate, 0.001 M EDTA) buffer. Restriction patterns were analysed using Bio-1D++ software (Vilber Lourmat) with a 12% homology coefficient. The similarity between the strains was determined automatically by specifying the formula of Nei & Li (1979). Strain clustering was performed using the unweighted pair group method with arithmetic means (Vilber Lourmat).

RESULTS AND DISCUSSION

Sixteen type strains of *Staphylococcus* species were analysed by 16S-ITS rRNA gene RFLP. All strains yielded a single amplification product, ranging in size from 1800 to 2000 bp, from the 16S-ITS rRNA gene region. Only *S. aureus* and *S. caprae* species yielded an extra DNA molecule, of size 1500 and 700 bp, respectively. RFLP profiles of the 16S-ITS rRNA gene were obtained using *TaqI* restriction enzyme digestion and analysed with a 12% homology coefficient using the Bio-1D++ program (Vilber Lourmat). Analysis of the *TaqI*

dendrogram resulted in 16 distinct RFLP haplotypes (Table 1, Fig. 1). *TaqI* digestion formed two main clusters, A and B, which displayed approximately 60% pattern homology (Fig. 1). Cluster A contained 13 of the haplotypes distributed into five subclusters (A1, A2, A3, A4 and A5; Fig. 1). The pattern

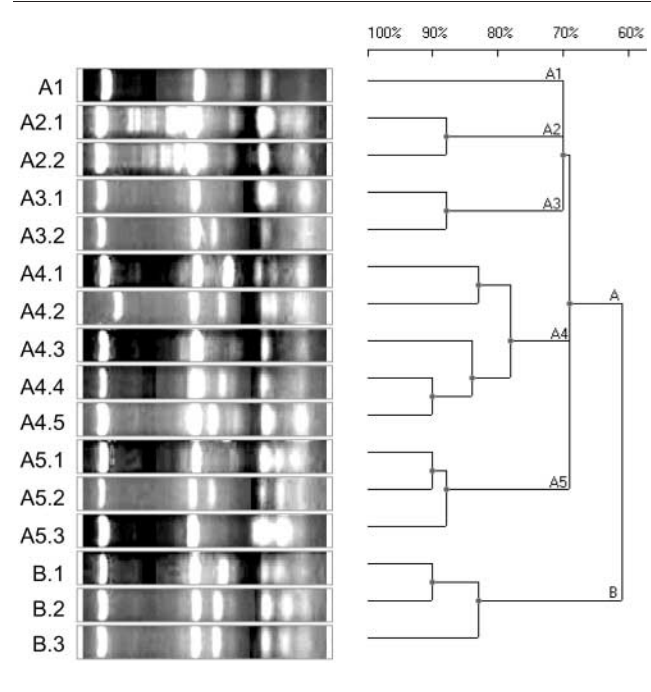


Fig. 1. Dendrogram of *TaqI* 16S-ITS rRNA gene RFLP haplotypes. A DNA size marker (O'RangeRuler 100 bp DNA ladder; MBI Fermentas) was used to normalize restriction profiles of the haplotypes. Restriction profiles were analysed with a 12% homology coefficient using Bio-1D++ software (Vilber Lourmat). The names of the clusters (right) and the haplotype groups (left) are indicated.

Table 1. 16S-ITS rRNA gene RFLP haplotype groups

RFLP haplotypes	Strain	No. tested
A1	<i>S. condimentii</i> (DSM 11674 ^T)	1
A2.1	<i>S. aureus</i> (ATCC 25923 ^T)	1
A2.2	<i>S. intermedius</i> (CCM 5739 ^T)	1
A3.1	<i>S. cohnii</i> subsp. <i>cohnii</i> (NRRL B-14756 ^T), Ege77	2
A3.2	<i>S. equorum</i> (NRRL B-14765 ^T)	1
A4.1	<i>S. auricularis</i> (NRRL B-14762 ^T)	1
A4.2	<i>S. chromogenes</i> (NRRL B-14759 ^T)	1
A4.3	<i>S. simulans</i> (NRRL B-14753 ^T)	1
A4.4	<i>S. lugdunensis</i> (NRRL B-14774 ^T)	1
A4.5	<i>S. caprae</i> (NRRL B-14757 ^T), JSCC2/22, Ege13	3
A5.1	<i>S. carnosus</i> (NRRL B-14760 ^T)	1
A5.2	<i>S. xylosum</i> (DSM 20266 ^T)	1
A5.3	<i>S. capitis</i> subsp. <i>capitis</i> (NRRL B-14752 ^T), OM2, Ege37	3
B.1	<i>S. epidermidis</i> (NRRL B-4268 ^T), RSKK 01015, KP15, AA-01, Ege104-20	5
B.2	<i>S. arlettae</i> (NRRL B-14764 ^T)	1
B.3	<i>S. gallinarum</i> (NRRL B-14763 ^T)	1

homology between these subclusters ranged from 69 to 70 %. In cluster A, the closest strains were *S. lugdunensis*/*S. caprae* (A4-4 and A4-5) and *S. carnosus*/*S. xylosus* (A5-1 and A5-2), showing approximately 90 % pattern homology. *S. condimentii* was the most distant of the cluster A strains (Fig. 1, A1). The A2 subcluster contained two coagulase-positive staphylococci (*S. aureus* and *S. intermedius*) with 88 % pattern homology. In addition, two novobiocin-resistant strains displayed 88 % pattern homology (*S. cohnii* subsp. *cohnii* and *S. equorum* in the A3 subcluster; Fig. 1). Cluster B contained only three haplotypes, and *S. epidermidis* and *S. arlettae* were found to be the closest strains within this cluster (Fig. 1). As expected, clinical isolates showed identical restriction patterns to the respective type strains (Table 1).

Species-level identification studies have often been based on sequencing of certain genes (Poyart *et al.*, 2001; Drancourt & Raoult, 2002) and hybridization studies (Goh *et al.*, 1997). In addition, PCR analyses of the length polymorphisms of the intergenic spacers residing between the 16S and 23S rRNA genes (Gurtler & Stanisich, 1996) or tRNA genes (Welsh & McClelland, 1992; Ehrenstein *et al.*, 1996) have also been important targets for the species-level identification of bacteria. tRNA gene intergenic-spacer-length polymorphism analysis of human staphylococci has differentiated 15 type strains (Maes *et al.*, 1997). RFLP of the 16S–23S rRNA gene intergenic spacer with *Dra*I has been applied for the identification of 31 *Staphylococcus* species (Mendoza *et al.*, 1998). In another study, amplification of ITS sequences per se has identified 29 different *Staphylococcus* species (Couto *et al.*, 2001). In these studies, the size of the amplicons has been much smaller than that of the 16S-ITS rRNA gene region. Some *Staphylococcus* species, especially *S. aureus* isolates, showing intraspecies polymorphism within the ITS sequence have also been reported (Mendoza *et al.*, 1998; Couto *et al.*, 2001). 16S-ITS rRNA gene RFLP of *S. aureus* isolates obtained from intensive care unit patients did not show any intraspecies polymorphism (Sudagidan *et al.*, unpublished results). Furthermore, *S. epidermidis* isolates from both human and animal (AA-01, isolated from the uterus of a horse) have displayed the same restriction patterns.

Recently, the 16S-ITS rRNA gene RFLP method has been successfully applied to the species-level identification of lactobacilli (Yavuz *et al.*, 2004a), thermophilic bacilli (Yavuz *et al.*, 2004b) and alkalophilic bacilli (Akbalik *et al.*, 2004). In the identification of lactobacilli, three restriction enzymes, *Alu*I, *Hae*III and *Taq*I, have been used. Among these enzymes, *Taq*I has been found to be the most discriminative (Yavuz *et al.*, 2004a). 16S-ITS rRNA gene RFLP with the *Hae*III enzyme, on the other hand, has identified both lactococci and enterococci much more efficiently than *Taq*I (Bulut *et al.*, 2005). However, this enzyme was not found to be useful for the differentiation of staphylococci (data not shown).

In the present study, all the type strains of 16 *Staphylococcus* species were differentiated as distinct RFLP haplotypes. The

remaining type strains of *Staphylococcus* should also be typed to assess fully the discriminatory power of 16S-ITS rRNA gene RFLP on staphylococci.

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