Evaluation of a ribosomal RNA gene probe for the identification of species and subspecies within the genus *Staphylococcus*

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To evaluate a 16S rRNA gene probe for the identification of staphylococcal species and subspecies, we have augmented previous studies involving 12 staphylococcal species by analysing the remaining 16 species currently classified in the genus *Staphylococcus*. *Hind*III- and *Eco*RI-restricted DNA of isolates from validly described species of *Staphylococcus* was probed with radiolabelled plasmid pBA2 containing 16S rDNA from *Bacillus subtilis*. The Dice coefficient was used to assess similarity between the 74 *Hind*III- and the 81 *Eco*RI-hybridization patterns obtained from a total of 271 isolates belonging to 31 staphylococcal taxa (28 species, of which three include two subspecies). The use of *Hind*III yielded a better discrimination of the staphylococci than the use of *Eco*RI. All of the isolates belonging to the same species or subspecies, except *S. hyicus* isolates, were recovered as homogeneous clusters using their *Hind*III hybridization patterns. The phenotypically close taxa were clearly distinguished. Thus, the method presented in this study constitutes a powerful tool for the identification of taxa within the genus *Staphylococcus*.

Introduction

In the 8th edition of *Bergey's Manual of Determinative Bacteriology*, the genus *Staphylococcus* consisted of three species (Baird-Parker, 1974). Currently, 28 species of *Staphylococcus* and ten subspecies are recognized on the basis of DNA–DNA hybridization (De la Fuente *et al.*, 1985; Kloos & Schleifer, 1986; Igimi *et al.*, 1989, 1990; Bannerman & Kloos, 1991; Kloos & Wolfshohl, 1991). Simplified identification schemes are regularly modified in order to discriminate between these taxa. However, because of the limited number of stable discriminating characteristics, many of the taxa remain difficult to distinguish from one another by phenotypic tests (Devriese *et al.*, 1985; Kloos & Schleifer, 1986; Hébert *et al.*, 1988; Igimi *et al.*, 1989; Brun *et al.*, 1990; Kloos & George, 1991). Several other approaches have been proposed in order to differentiate between staphylococcal species. The methods include: numerical taxonomy (Goodfellow *et al.*, 1983; Gunn *et al.*, 1983; Hoover *et al.*,

1983); cell wall peptidoglycan and teichoic acid composition (Endl *et al.*, 1983; Schumacher-Perdreau *et al.*, 1983); bacteriolytic activity patterns (Varaldo & Satta, 1978); serotyping (Pillet & Orta, 1981); electrophoretic comparison of isofunctional enzymes (Götz & Schleifer, 1976; Zimmerman & Kloos, 1976; Zimmerman, 1976); immunological relatedness of catalases (Schleifer *et al.*, 1979) and fructose-1,6-diphosphate aldolases (Fischer *et al.*, 1983); gel electrophoresis of cellular proteins (Clink & Pennington, 1987; Pennington *et al.*, 1991) and penicillin-binding proteins (Fontana *et al.*, 1981; Pierre *et al.*, 1990); fatty acid profiles (O'Donnell *et al.*, 1985); Fourier-transform infrared spectroscopy patterns (Helm *et al.*, 1991); and endonuclease-cleaved cellular DNA (Bialkowska-Hobrzanska *et al.*, 1990a) and rRNA gene polymorphisms (De Buyser *et al.*, 1989; Thomson-Carter *et al.*, 1989; El Solh *et al.*, 1990; Bialkowska-Hobrzanska *et al.*, 1990b; Pennington *et al.*, 1991). Since these methods have been used on only a limited number of staphylococcal species, their ability to discriminate between all species is uncertain. The method used in the current study is based on rRNA gene polymorphisms (De Buyser *et al.*, 1989) using pBA2 (Iglesias *et al.*, 1983) containing 16S rDNA of *Bacillus subtilis* as a probe and *Hind*III and *Eco*RI to cleave the cellular DNA from a

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Abbreviation: HP, hybridization patterns.

comprehensive range of staphylococci. We have thus augmented two previous studies involving 14 staphylococcal taxa including 12 species and five subspecies (De Buyser *et al.*, 1989; El Solh *et al.*, 1990) by analysing the remaining 17 taxa including 16 species and one subspecies.

Methods

Bacterial isolates. The 75 epidemiologically unrelated isolates studied came from human (16 isolates), animal (30 isolates), food derived from animals (26 isolates) and unknown (3 isolates) sources (Table 1). These isolates represent 17 staphylococcal taxa including 16 species and one subspecies. The isolates chosen were those which could be assigned to

Table 1. *Characteristics of the 75 staphylococcal isolates*

Taxon and strain designation	Source*	Isolated from:	<i>Hind</i> III hybridization pattern	<i>Eco</i> RI hybridization pattern
<i>S. aureus</i>				
subsp. <i>anaerobius</i>				
ATCC 35844†	K. H. Schleifer	Sheep	H99	E99
MVF 25			H99	E99
MVF 39			H99	E99
MVF 41			H99	E99
MVF 43			H99	E99
<i>S. delphini</i>				
ATCC 49171†	P. E. Varaldo	Dolphin	H900	E900
ATCC 49172			H900	E900
<i>S. schleiferi</i>				
ATCC 43808†	J. Freney	Man	H120	E120
88193	FNRCs		H121	E121
88271			H121	E121
82558		Unknown	H121	E121
<i>S. capitis</i>				
ATCC 27840†	W. E. Kloos	Man	H130	E130
HM 11			H130	E130
75768	FNRCs		H130	E130
78008			H130	E130
86163			H131	E131
<i>S. auricularis</i>				
ATCC 33753†	W. E. Kloos	Man	H140	E140
ATCC 33750			H140	E140
BB 1	M. L. De Buyser	Goat's milk	H140	E140
CH 44			H141	E141
<i>S. simulans</i>				
ATCC 27848†	W. E. Kloos	Man	H100	E100
78078	FNRCs		H100	E100
77398			H100	E101
MAW 222	W. E. Kloos		H100	E102
CH 38	M. L. De Buyser	Goat's milk	H101	E103
CH 137			H101	E104
CH 235			H102	E105
CH 173			H102	E105
CH 154			H102	E105
<i>S. felis</i>				
ATCC 49168†	S. Igimi	Cat	H920	E920
GD 104			H921	E921
<i>S. carnosus</i>				
DSM 20501†	DSM	Meat	H110	E110
TM 300	F. Götz		H110	E110
PCA 43			H110	E110
PCT 20			H110	E110
<i>S. caprae</i>				
CCM 3573†	L. A. Devriese	Goat's milk	H800	E800
141-15			H800	E801
CH 55			H800	E801
CH 150	M. L. De Buyser		H800	E801
CH 244			H800	E801
CH 116			H800	E801
CH 121			H800	E801

Table 1.—(continued).

Taxon and strain designation	Source*	Isolated from:	HindIII hybridization pattern	EcoRI hybridization pattern
<i>S. caseolyticus</i>				
ATCC 13548†	CIP	Milk	H190	E190
CH 18	M. L. De Buyser	Goat's milk	H190	E191
<i>S. saccharolyticus</i>				
DSM 20359†	CIP	Unknown	H195	E195
<i>S. equorum</i>				
DSM 20674†	L. A. Devriese	Horse	H38	E35
PA 209			H38	E35
PA 219			H38	E35
PA 235			H38	E35
CH 65	M. L. De Buyser	Goat's milk	H38	E35
78037	FNRCS	Horse	H38	E34
CH 100	M. L. De Buyser	Goat's milk	H39	E36
<i>S. sciuri</i>				
ATCC 29062†	W. E. Kloos	Squirrel	H810	E810
ATCC 29068		Unknown	H810	E810
ATCC 29059		Opossum	H810	E810
ATCC 29060		Man	H811	E811
CH 231	M. L. De Buyser	Goat's milk	H812	E812
<i>S. lentus</i>				
ATCC 29070†	W. E. Kloos	Goat	H820	E820
K 12			H820	E820
K 15			H820	E820
CH 174	M. L. De Buyser	Goat's milk	H820	E820
CH 95			H820	E820
CH 224			H820	E820
CH 250			H820	E821
<i>S. gallinarum</i>				
CCM 3372†	L. A. Devriese	Poultry	H150	E150
P 21			H151	E151
P 57			H151	E152
<i>S. arlettae</i>				
DSM 20672†	L. A. Devriese	Poultry	H160	E160
DSM 20673			H160	E160
BP 32			H160	E160
BP 36			H160	E160
<i>S. kloosii</i>				
DSM 20676†	L. A. Devriese	Animal	H170	E170
DM 93			H171	E171
SC 220			H172	E172
PI 427			H173	E173

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† Type strain.

known staphylococcal taxa according to published identification schemes (Devriese *et al.*, 1985; De la Fuente *et al.*, 1985; Kloos & Schleifer, 1986; Freney *et al.*, 1988; Varaldo *et al.*, 1988; Igimi *et al.*, 1989). The biochemical tests used were those previously described (De Buyser *et al.*, 1989).

Isolation and labelling of plasmid DNA. Plasmids pBR322 (Bolivar *et al.*, 1977) and pBA2 (Iglesias *et al.*, 1983) were used as probes in the

hybridization experiments. Plasmid pBA2 contains a 2.3 kb *Bacillus subtilis* DNA fragment encoding 16S rRNA inserted in the *Hind*III site of pBR322. Plasmids were isolated, purified and labelled with [α - 32 P]dCTP (110 TBq mmol $^{-1}$) as previously described (De Buyser *et al.*, 1989).

Gel electrophoresis of endonuclease-cleaved DNA, Southern transfer and hybridization. Extraction of cellular DNA from staphylococcal isolates,

Fig. 1. Autoradiogram of *Hind*III-digested cellular DNA from 13 staphylococcal strains, separated by electrophoresis and probed with α - 32 P-labelled plasmid pBA2. Lanes A to D, *S. aureus* subsp. *anaerobius* strains ATCC 35844†, MVF 25, MVF 39, MVF 41; lane E, *S. caprae* strain CCM 3573†; lanes F to G, *S. caseolyticus* strains ATCC 13548†, CH 18; lane H, *S. saccharolyticus* strain DSM 20359†; lane I, *S. simulans* strain CH 235; lanes J to M, *S. carnosus* strains DSM 20501†, TM 300, PCA 43, PCT 20. † Type strain.

cleavage with restriction endonucleases *Hind*III or *Eco*RI, separation by electrophoresis, blotting on nylon membranes of DNA restriction fragments and hybridization with the probes were carried out as previously described (De Buyser *et al.*, 1989). The molecular size marker used was a Raoul I ladder (Appligène, France).

Comparison of hybridization patterns (HP). The sizes of the bands appearing in the HP of the isolates included in this study and of those described in our previous studies (De Buyser *et al.*, 1989; El Solh *et al.*, 1990) were entered on a IBM/PS2 computer. The similarity between any two HP was evaluated according to the Dice coefficient (Dice, 1945).

Clustering of HP was based on the centroid linkage algorithm [BMDP 2M statistical program (1990), University of California Press, Berkeley, USA]. This program joins HP and/or clusters of HP in a stepwise manner until all cases are combined into one cluster. The algorithm uses the distance between the centres of each cluster as a criterion for joining (amalgamating) clusters.

Results

Each of the *Hind*III- or *Eco*RI-cleaved staphylococcal cellular DNAs was probed with pBR322. No restriction fragments hybridized with pBR322; hence, pBA2 was used as a probe for the visualization of rRNA genes without separating the 16S rDNA insert from the pBR322 vector.

Using pBA2 as a probe, the 75 staphylococcal isolates belonging to 17 staphylococcal taxa (16 species and one subspecies) (Table 1) yielded 30 distinct *Hind*III HP and 38 distinct *Eco*RI HP. Fig. 1 shows the *Hind*III HP of 13 isolates belonging to six distinct taxa. HP are schematically represented in Figs 2 and 3. Different patterns were

Fig. 2. Schematic representation of the 30 *Hind*III-HP detected among the 75 staphylococcal isolates studied. Dashed lines indicate very weakly labelled bands. The patterns marked with an asterisk (*) are those corresponding to the type strain of each taxon.

observed for the isolates belonging to distinct taxa (species and subspecies). One to four *Hind*III HP and one to six *Eco*RI HP were detected within each taxon.

The 74 *Hind*III HP and 81 *Eco*RI HP obtained from 271 isolates belonging to 28 species and six subspecies (31 taxa in all), including the 75 isolates described in this study (Table 1) and the 196 previously described independent isolates (De Buyser *et al.*, 1989; El Solh *et al.*, 1990) were compared.

Each of the 31 staphylococcal taxa analysed was characterized by a core of common bands hybridizing with pBA2. For each taxon, the number of common

bands varied with the restriction enzyme used. With *Eco*RI-digested DNA, the taxon-specific core consisted of three bands for *S. aureus*, and five to ten bands for each of the remaining 30 taxa. With *Hind*III-digested DNA, the taxon-specific pattern consisted of four bands for *S. aureus*, *S. intermedius* subsp. *carnivora*, *S. hyicus*, *S. schleiferi*, *S. caseolyticus* and *S. cohnii* subsp. *cohnii*, and five to ten bands for each of the remaining 25 taxa. Interestingly, the taxon-specific patterns for taxa which are difficult to distinguish from each other using phenotypic identification schemes, for example *S. simulans* and *S. felis*, *S. sciuri* and *S. lentus*, *S. xylosus*

Fig. 3. Schematic representation of the 38 *Eco*RI-HP detected among the 75 staphylococcal isolates studied. Dashed lines indicate very weakly labelled bands. The patterns marked with an asterisk (*) are those corresponding to the type strain of each taxon.

Table 2. Percentage similarity (%S) of the hybridization patterns (HP) observed among 271 staphylococcal isolates using pBA2 as a probe

The percentage similarity, %S, was calculated by using the Dice coefficient.

Taxon	No. of isolates	HindIII-hybridization patterns			EcoRI-hybridization patterns		
		No. of intra-taxon HP	%S: lowest intra-taxon values*	%S: highest inter-species values†	No. of intra-taxon HP	%S: lowest intra-taxon values*	%S: highest inter-species values†
<i>S. aureus</i> subsp. <i>aureus</i>	78	10	61.5	54.5 (54.5)	15	62.5	58.8 (70.5)
<i>S. aureus</i> subsp. <i>anaerobius</i>	5	1		37.5 (54.5)	1		70.5 (70.5)
<i>S. intermedius</i> pigeon	1	1		66.6 (83.3)	1		42.8 (26.6)
<i>S. intermedius</i> carnivora	6	3	72.7	60.0 (83.3)	2	88.8	55.5 (26.6)
<i>S. delphini</i>	2	1		66.6	1		50.0
<i>S. hyicus</i>	6	4	61.5	62.5	4	55.5	55.5
<i>S. chromogenes</i>	6	2	92.3	66.6	1		44.4
<i>S. schleiferi</i>	4	2	72.7	66.6	2	88.8	42.1
<i>S. lugdunensis</i>	8	2	94.7	52.6	3	84.2	50.0
<i>S. hominis</i>	5	1		52.6	1		62.5
<i>S. warneri</i>	11	2	93.3	40.0	1		50.0
<i>S. haemolyticus</i>	6	3	66.6	52.6	2	75.0	62.5
<i>S. epidermidis</i>	50	9	66.6	62.5	4	80.0	53.3
<i>S. capitis</i>	5	2	95.2	50.0	2	84.2	66.6
<i>S. auricularis</i>	4	2	88.8	40.0	2	94.1	58.8
<i>S. simulans</i>	9	3	75.0	58.8	6	63.1	52.6
<i>S. felis</i>	2	2	93.3	52.6	2	83.3	28.5
<i>S. carnosus</i>	4	1		58.8	1		52.6
<i>S. caprae</i>	7	1		44.4	2	75.0	58.8
<i>S. caseolyticus</i>	2	1		28.5	2	77.7	50.0
<i>S. saccharolyticus</i>	1	1		44.4	1		42.8
<i>S. saprophyticus</i>	7	1		60.0	1		55.5
<i>S. cohnii</i> subsp. <i>cohnii</i>	3	2	61.5	30.7 (14.2)	2	90.0	63.1 (77.7)
<i>S. cohnii</i> subsp. <i>urealyticum</i>	4	2	82.3	63.1 (14.2)	4	70.5	66.6 (77.7)
<i>S. xylosum</i>	5	2	66.6	63.1	2	88.8	66.6
<i>S. equorum</i>	7	2	92.3	35.2	3	90.0	63.1
<i>S. sciuri</i>	5	3	72.7	45.4	3	66.6	52.6
<i>S. lentus</i>	7	1		45.4	2	94.1	42.2
<i>S. gallinarum</i>	3	2	85.7	60.0	3	66.6	66.6
<i>S. arlettae</i>	4	1		18.1	1		58.8
<i>S. kloosii</i>	4	4	63.1	50.0	4	62.5	63.1

* No value is reported when a single HP was observed within a taxon.

† The values in parentheses correspond to the inter-subspecies values within one species.

Fig. 4. Classification of the 74 *Hind*III-HP observed for the 271 isolates belonging to 31 staphylococcal taxa (28 species and six subspecies). Similarity between patterns was measured by using the Dice coefficient. Clustering was performed by a centroid linkage algorithm (BMDP 2M program). The horizontal axis represents the distance between patterns expressed as a percentage of the greatest amalgamation distance (0-909) found between patterns in this dendrogram. For each HP the number of strains is indicated in parentheses.

and *S. equorum*, *S. aureus* and *S. intermedius*, *S. warneri* and *S. hominis* and *S. hyicus* and *S. chromogenes*, were clearly distinct (Figs. 2 and 3; De Buyser *et al.*, 1989).

The Dice coefficient was used to assess the similarity between the 74 *Hind*III HP and the 81 *Eco*RI HP

detected among the 271 staphylococci. The taxa which may be identified by this method are those for which the lowest intra-taxon percentage of similarity (%S) is higher than the highest inter-taxon %S. The values obtained are reported in Table 2. Using *Hind*III- and *Eco*RI-digested

DNA, 29 and 26 taxa could be distinguished, respectively.

The confidence in an identification obtained using this method increases when the difference between the lowest intra-taxon %S and the highest inter-taxon %S is great. Similar discrimination was observed with *Hind*III and *Eco*RI for nine taxa, but for the remaining 22 taxa the ability to distinguish taxa varied with the enzyme used. Fourteen taxa were separated more clearly when *Hind*III was used to cleave the cellular DNA, whereas eight taxa were distinguished better with *Eco*RI. The two subspecies of *S. intermedius* could only be separated from each other with *Eco*RI digests, whereas *S. aureus*, *S. cohnii* subsp. *urealyticum*, *S. gallinarum* and *S. kloosii* could only be distinguished with *Hind*III digests. Neither of these enzymes permitted the identification of *S. hyicus*.

The dendrogram (Fig. 4) shows the relationships between the 74 *Hind*III HP detected. All *Hind*III HP detected within the same taxon, except *S. hyicus* HP, were homogeneously clustered, showing an intra-taxon amalgamation distance varying from 0% to 38%. Phenotypically close taxa such as *S. warneri* and *S. hominis*, *S. simulans* and *S. felis*, *S. aureus* and *S. intermedius*, *S. xylosus* and *S. equorum*, *S. arlettae* and *S. gallinarum* were separated by amalgamation distances of at least 65%. The two subspecies delineated within *S. cohnii* and *S. aureus* were also separated by high distances ($\geq 83\%$).

Discussion

The method based on rRNA gene restriction fragment length polymorphisms has been proposed for discriminating between species of *Staphylococcus* (De Buyser *et al.*, 1989; Thomson-Carter *et al.*, 1989; El Solh *et al.*, 1990; Bialkowska-Hobrzanska *et al.*, 1990b; Pennington *et al.*, 1991). In this study, we report the comparative analysis of the *Hind*III- and *Eco*RI-HP of cellular DNA from 271 isolates belonging to 31 staphylococcal taxa (28 species, six subspecies), using a cloned 2.3 kb DNA fragment encoding 16S rRNA from *Bacillus subtilis* as a probe (Iglesias *et al.* 1983). Thomson-Carter *et al.* (1989) studied 22 isolates belonging to seven species, using *Hind*III and *Eco*RI to cleave cellular DNA and 16S + 23S rRNA from *E. coli* as a probe. Bialkowska-Hobrzanska *et al.* (1990b), using the same probe, studied 78 strains belonging to 15 species after cleavage of cellular DNA with *Cla*I. In each of these studies, a taxon-specific core of common bands was detected in the HP. These cores varied from one study to another because of the use of distinct probes and restriction enzymes. In the present study, all of the currently described staphylococcal species were analysed using the same probe and

endonucleases, thus creating a database for the accurate identification of any staphylococcal isolate.

To evaluate the discriminating ability of the method, it was necessary to calculate the lowest intra-taxon- and the highest inter-taxon-%S between the HP for each taxon and for each of the two enzymes tested. The use of *Hind*III yielded a better discrimination of most of the staphylococcal taxa. However, the two subspecies of *S. intermedius* could only be separated using *Eco*RI. Moreover, *S. hyicus* could not be easily distinguished either with *Hind*III or with *Eco*RI. Grimont & Grimont (1986) have shown that divergence in DNA-DNA hybridization within the species *Salmonella* or *Serratia* resulted in significant intra-taxon diversity of patterns. DNA-DNA hybridization (Devriese *et al.*, 1978; Kloos, 1980), numerical taxonomy (Hoover *et al.*, 1983) as well as miniaturized and conventional identification methods (Lmmler, 1991) showed a divergence between *S. hyicus* isolates obtained from phylogenetically distant hosts. Heterogeneity observed within *S. hyicus* HP may therefore be attributed to the diversity of hosts (pig, horse, poultry, cow) of the isolates we examined in this study. Nevertheless, *S. hyicus* HP could clearly be distinguished from the HP detected within the phenotypically close taxa *S. aureus*, *S. intermedius* and *S. chromogenes*.

The method proposed in this study is regularly used in our Reference Centre when the use of simple phenotypic identification schemes fails to identify a staphylococcal isolate to any of the described species. The HP obtained with distinct DNA preparations and independent agarose gel electrophoresis are reproducible. For each unknown isolate, the sizes of the *Eco*RI and *Hind*III hybridizing bands are compared with those obtained previously (De Buyser *et al.*, 1989; El Solh *et al.*, 1990) and in the present study. Since this method appears to be limited by the lack of precision with which the sizes of the hybridizing bands are evaluated, the sizes are measured by comparison not only with those of a Raoul I ladder but also with those of the HP obtained from staphylococcal type strains belonging to the most phenotypically close taxa, run side-by-side on the same gel. Non-radioactive labelling of the probes provides better resolution between the hybridization bands (Altwegg & Mayer, 1989) and is now being tested to improve the accuracy of size measurements.

In conclusion, the method presented in this study constitutes a powerful tool for the identification of staphylococcal isolates. Taxa which are difficult to separate using phenotypic tests are clearly distinguished. This method cannot replace quantitative DNA-DNA hybridization studies for estimating genomic relationships between staphylococci. However, it does permit the rapid screening of new taxa.

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