The value of rRNA gene restriction site polymorphism analysis for delineating taxa in the genus *Staphylococcus*

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A total of 101 staphylococcal strains were ribotyped using *Eco*RI and *Hin*dIII as restriction enzymes and plasmid pBA2 as the rDNA probe. Isolates from 10 newly described staphylococcal taxa were among those examined. All the ribotypes were added to our database, Staph DB, which now contains the sizes of the bands of 135 *Eco*RI and 120 *Hin*dIII ribotypes from 408 strains belonging to 42 staphylococcal taxa. The relatedness of ribotypes was evaluated by using the Dice coefficient. The ribotypes, and thus the strains, were clustered by the unweighted pair group method with averages (UPGMA). Separation into clusters correlated well with the delineation of the staphylococcal species but not with that of the different subspecies. No discrimination was possible between *Staphylococcus vitulinus* and *Staphylococcus pulvereri*. Ecovar-specific groups were evident within *Staphylococcus intermedius* and *Staphylococcus hyicus*. The data increase the usefulness of rRNA gene restriction site polymorphism analysis for staphylococcal taxonomy.

Keywords: *Staphylococcus*, ribotype, classification

INTRODUCTION

There has been much effort made over recent years to classify, identify and type staphylococci isolated from human, animal and environmental samples. Nearly all of the methods used for these investigations are based on the study of the genome. Quantitative DNA pairing and 16S rRNA sequencing are required for classification. Species-specific DNA-based assays are used for identification with either oligonucleotides for PCR experiments or probes for colony hybridization. Random amplification of various DNA fragments, sequencing of the few variable DNA regions and/or analysis of the cellular DNA restriction patterns with or without hybridization with probes consisting of mobile element sequences are used for epidemiological surveys. rDNA fingerprinting in staphylococci has been suggested to be appropriate for distinguishing between taxa and for typing strains (Gaszewska-Mastalarz *et al.*, 1998; Hesselbarth & Schwarz, 1995; Kluytmans *et al.*, 1998; Monzon-Moreno *et al.*, 1991; Thomson-Carter *et al.*, 1989; Walcher-Salesse *et al.*, 1992). This method, commonly known as ribotyping, is based on targeting parts of the genome, i.e. rRNA operons, that are conserved, present in multiple copies and easily detectable either by amplification with universal primers (Forsman *et al.*, 1997; Gaszewska-Mastalarz *et al.*, 1998; Mendoza *et al.*, 1998) or by hybridization with universal probes (Bialkowska-Hobrzanska *et al.*, 1990; Chesneau *et al.*, 1992; De Buyser *et al.*, 1989, 1992; Hesselbarth & Schwarz, 1995; Irlinger *et al.*, 1997; Pennington *et al.*, 1991; Thomson-Carter *et al.*, 1989). Further comparisons of staphylococcal strains require that the details of the corresponding ribotypes be stored in databases. The performance of this approach for the assignment of atypical strains to defined taxa (Chesneau *et al.*, 1992) and the detection of strains belonging to undescribed taxa (Chesneau *et al.*, 1993b; Kloos *et al.*, 1997; Webster *et al.*, 1994) is dependent largely on the continuous updating of the databases as new taxa are described and strains belonging to previously unknown ecovars are isolated.

Thirty-five species and nine subspecies are currently recognized in the genus *Staphylococcus* (Kloos *et al.*, 1998a, b; Lambert *et al.*, 1998; Probst *et al.*, 1998).

Abbreviations: HP, hybridization pattern; UPGMA, unweighted pair group method with averages.

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Table 1. Characteristics of the 101 staphylococcal strains studied

New ribotypes are underlined.

Taxon	Strain	Host (origin of the sample)	*Eco*RI HP	*Hin*dIII HP
S. capitis subsp. *ureolyticus*	ATCC 49324	Human (external auditory meatus)	E131	H135
	ATCC 49325	Human (inguinal area)	E131	H137
	ATCC 49326^{T}	Human (scalp)	E131	H135
	ATCC 49327	Human (inguinal area)	E131	H130
	DLB 8531	Human (scalp)	E131	H135
	1436-6	Human (forehead)	E136	H130
	MK8731	Human (anterior nares)	E135	H130
S. lutrae	DSM 10244^{T}	Otter (mammary gland)	E950	H950
	DSM 10245	Otter (liver abscess)	E951	H951
S. muscae	CCM 4175^{T}	Fly (cow shed)	E180	H180
	CCM 4176	Fly (cow shed)	E180	H180
	CCM 4177	Fly (cow shed)	E180	H180
	CCM 4177	Fly (cow shed)	E181	H181
S. piscifermentans	SK03^{T}	Fermented shrimp	E910	H910
	SK14	Fermented fish	E910	H910
	SK02	Fermented shrimp	E911	H911
	SK04	Fermented shrimp	E911	H911
	SK15	Fermented fish	E910	H910
	Ph79S	Fermented fish	E912	H912
	SK05	Fermented shrimp	E913	H913
	Ph79L	Fermented fish	E912	H912
	SK16	Fermented fish	E914	H910
	Ph78	Fermented fish	E912	H912
S. pulvereri	PCM 2443^{T}	Hip infection	E943	H942
	PCM 2457	Human (unknown)	E944	H942
	PCM 2458	Human (unknown)	E944	H942
	PCM 2459	Human (unknown)	E944	H942
	PCM 2460	Carcass of diseased chicken	E944	H942
S. saprophyticus subsp. *bovis*	CCM 4410^{T}	Cow (anterior nares)	E31	H185
	KV20	Cow (anterior nares)	E31	H35
	KV30	Cow (anterior nares)	E31	H35
	CCM 4411	Cow (anterior nares)	E31	H185
S. schleiferi subsp. *coagulans*	GA400	Dog (otitis specimen)	E125	H125
	GA238	Dog (otitis specimen)	E126	H126
	GA11	Dog (otitis specimen)	E126	H126
	GA288	Dog (otitis specimen)	E126	H126
	GA390	Dog (otitis specimen)	E127	H126
S. sciuri subsp. *carnaticus*	DD791^{T}	Leg of veal, sliced	E813	H813
	BL2	Beef cheek	E814	H813
	B3L6	Aberdeen Angus bull	E814	H813
S. sciuri subsp. *rodentium*	R1-33^{T}	Norway rat	E815	H810
	CR5-5	Cotton rat	E816	H810
	GMWg14	Pilot whale	E815	H814
S. vitulinus	ATCC 51145^{T}	Ground lamb	E940	H940
	VE15	Raw veal trimmings	E942	H941
	VE23	Raw veal trimmings	E941	H941
	V51	Pine vole	E942	H942

Table 1 *(cont.)*

Taxon	Strain	Host (origin of the sample)	*Eco*RI HP	*Hin*dIII HP
S. hyicus	BM10325	Cattle	E930	H930
	BM10326	Cattle	E930	H930
	BM10327	Cattle	E930	H930
	BM10328	Cattle	E931	H931
	BM10329	Cattle	E930	H930
	BM10330	Cattle	E930	H930
	BM10331	Cattle	E930	H930
	BM10332	Cattle	E930	H930
	BM10333	Cattle	E931	H932
	BM10334	Cattle	E931	H932
	BM10335	Cattle	E931	H932
	BM10336	Cattle	E931	H931
	BM10337	Cattle	E931	H932
	BM10338	Cattle	E932	H935
	BM10339	Pig	E933	H13
	BM10340	Pig	E16	H13
	BM10341	Pig	E16	H13
	BM10342	Pig	E16	H13
	BM10343	Pig	E16	H13
	BM10344	Pig	E16	H934
	BM10345	Pig	E16	H13
	BM10346	Pig	E16	H13
	BM10347	Pig	E16	H13
	BM10348	Pig	E16	H933
	BM10350	Pig	E934	H13
	BM10351	Pig	E16	H13
	BM10352	Pig	E933	H13
	BM10353	Pig	E933	H13
S. intermedius	BM10355	Dog	E873	H12
	BM10356	Dog	E15	H12
	BM10357	Dog	E15	H12
	BM10358	Dog	E873	H12
	BM10359	Dog	E874	H874
	BM10360	Dog	E15	H12
	BM10361	Dog	E15	H12
	BM10362	Dog	E875	H874
	BM10363	Dog	E876	H872
	BM10364	Dog	E877	H12
	BM10365	Dog	E15	H870
	BM10366	Dog	E878	H870
	BM10367	Dog	E15	H871
	BM10368	Dog	E879	H873
	BM10369	Dog	E870	H874
	BM10370	Dog	E15	H873
	BM10371	Dog	E871	H873
	BM10372	Dog	E15	H12
	BM10373	Dog	E15	H12
	BM10374	Dog	E15	H12
	BM10375	Dog	E870	H11
	BM10376	Dog	E872	H872
	BM10902	Pigeon	E860	H875
	BM10903	Pigeon	E860	H875
	BM10904	Pigeon	E861	H12
	BM10905	Pigeon	E862	H874

Our database of ribotypes, Staph DB, was set up with 307 staphylococcal strains belonging to 32 taxa (Chesneau *et al.*, 1992; De Buyser *et al.*, 1989, 1992). We report an analysis of the ribotypes of a further 101 staphylococcal isolates. The aims of our analysis were to update Staph DB and to assess whether the diversity of the ribotypes within *Staphylococcus intermedius* and *Staphylococcus hyicus* corresponds to the distribution of the isolates in various ecovars. The 101 isolates were as follows: (i) 47 strains belonging to *Staphylococcus lutrae* (Foster *et al.*, 1997), *Staphylococcus muscae* (Hájek *et al.*, 1992), *Staphylococcus piscifermentans* (Tanasupawat *et al.*, 1992), *Staphylococcus pulvereri* (Zakrzewska-Czerwińska *et al.*, 1995), *Staphylococcus vitulinus* (Webster *et al.*, 1994), *Staphylococcus schleiferi* subsp. *coagulans* (Igimi *et al.*, 1990), *Staphylococcus capitis* subsp. *ureolyticus* (Bannerman & Kloos, 1991), *Staphylococcus saprophyticus* subsp. *bovis* (Hájek *et al.*, 1996), *Staphylococcus sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *rodentium* (Kloos *et al.*, 1997); and (ii) 54 strains belonging to various different ecovars of *S. hyicus* and *S. intermedius*.

METHODS

Bacterial strains. The relevant characteristics of the 101 staphylococcal strains analysed here are reported in Table 1. Forty-seven strains were sent to the French National Reference Centre for Staphylococci by the laboratories that characterized the species and subspecies. The remaining strains belonged either to *S. hyicus* (28 strains) or to *S. intermedius* (26 strains). All were examined phenotypically by standard laboratory procedures. Key characters, such as the presence of catalase and oxidase activities, the production of extracellular coagulase and thermonuclease enzymes as well as cell clumping, were tested as described previously (Chesneau *et al.*, 1993b). Acid production from carbohydrates and additional biochemical traits were determined using the ID32 Staph micro method (Brun *et al.*, 1990). Xylose utilization was tested on agar plates (Kloos *et al.*, 1974).

Ribotyping. Total cellular DNA from staphylococcal strains was cleaved with *Eco*RI and *Hin*dIII, used independently (Amersham). The resulting DNA fragments were separated by electrophoresis on 0·7% (w/v) agarose gels and transferred onto Hybond-N$^+$ membranes (Amersham). The 16S rDNA probe was labelled with ECL-Probe Amp reagents (Amersham) using pBA2 as the template (Iglesias *et al.*, 1983) and oligodeoxynucleotides An1 (5′-CTCATGTTTGACAGCTTATCATCG-3′, 24-mer) and An2 (5′-GCAATTTAACTGTGATAAACTACCGC-3′, 26-mer) as primers. The hybridization experiments were carried out at 68 °C. Autoradiography film (Amersham) was exposed to gels for 5 min.

Data processing. The sizes of the bands appearing in the *Eco*RI and *Hin*dIII hybridization patterns (HP) were calculated by classical log regression analysis. Each new HP was registered as a new ribotype and then introduced into the database, Staph DB, with the designation of the restriction enzyme. Similarity between pairs of ribotypes was scored according to the Dice coefficient (Dice, 1945). From a distance matrix in PHYLIP format (Felsenstein, 1993), UPGMA clustering and dendrogram drawing were done by the TAXOTRON software (Grimont, 1998).

RESULTS

The phenotypic traits of the 101 strains tested were determined and compared with those reported in the papers describing the species and subspecies. Our findings for the five strains described as belonging to *S. pulvereri* were not consistent with those reported previously (Zakrzewska-Czerwińska *et al.*, 1995). Indeed, as in another study (Petráš, 1998), the five *S. pulvereri* strains appeared phenotypically similar to *S. vitulinus*. In our hands, aesculin hydrolysis was the only trait that enabled phenotypic differentiation between the two species: the four *S. vitulinus* strains tested hydrolysed aesculin whereas the five *S. pulvereri* strains did not.

The ribotypes of the 101 strains tested were determined. There were 41 *Eco*RI ribotypes and 29 *Hin*dIII ribotypes that were not previously present in Staph DB (Table 1). The other ribotypes were indistinguishable from those already characterized (Chesneau *et al.*, 1992; De Buyser *et al.*, 1989, 1992). Species not previously represented in Staph DB consistently gave new ribotypes, with the exception of *S. pulvereri* and *S. vitulinus*. However, strains belonging to different subspecies in some cases shared a single ribotype. The animal strains of *S. saprophyticus* subsp. *bovis* exhibited the same *Eco*RI ribotype (E31 HP) as the human strains of *S. saprophyticus* subsp. *saprophyticus*. Three strains of *S. capitis* subsp. *ureolyticus* and the type strain of *S. capitis* subsp. *capitis* shared the H130 HP. Two strains of *S. sciuri* subsp. *rodentium* and the type strain of *S. sciuri* subsp. *sciuri* shared the H810 HP (Table 1).

The Dice coefficient and UPGMA clustering were used to compare the 94 *Eco*RI ribotypes and the 91 *Hin*dIII ribotypes found for the 307 staphylococcal strains examined previously in our laboratory (Chesneau *et al.*, 1992; De Buyser *et al.*, 1989, 1992) with those of the 101 strains studied here. Two dendrograms were constructed on the basis of the levels of similarity between the ribotypes obtained after cleavage with *Eco*RI (Fig. 1) or with *Hin*dIII (Fig. 2). For both enzymes, a 62% similarity cut-off value gave the best species-specific clustering of the strains. Using this cut-off value, (i) the strains belonging to *S. vitulinus* and *S. pulvereri* fell into a single cluster, (ii) the strains belonging to each of *S. hyicus* and *S. intermedius* were distributed into more than one cluster and (iii) the strains belonging to all but one subspecies were not grouped into separate clusters. Excluding these four species, *Eco*RI allowed better discrimination of the staphylococcal species than did *Hin*dIII. Using *Eco*RI, only *Staphylococcus kloosii* could not be indi-

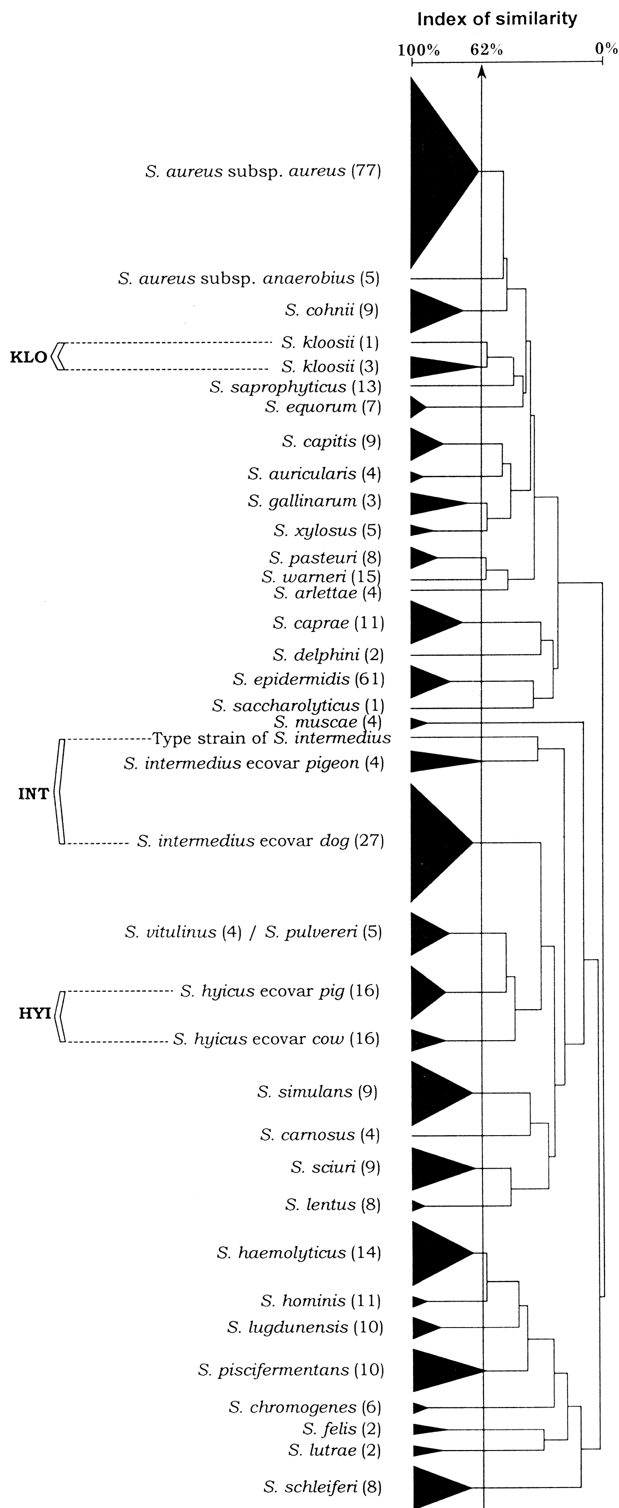

Fig. 1. Dendrogram showing the relatedness of the 135 *Eco*RI ribotypes included in Staph DB (cluster analysis by the Dice coefficient and UPGMA). The clusters are represented by triangles, the lengths of the bases of which are proportional to the number of ribotypes (the smallest correspond to two ribotypes, e.g. *Staphylococcus auricularis*). The number of strains tested is given in parentheses. Taxa, strains of which are not clustered, are indicated on the left: HYI, *S. hyicus*; INT, *S. intermedius*; KLO, *S. kloosii*.

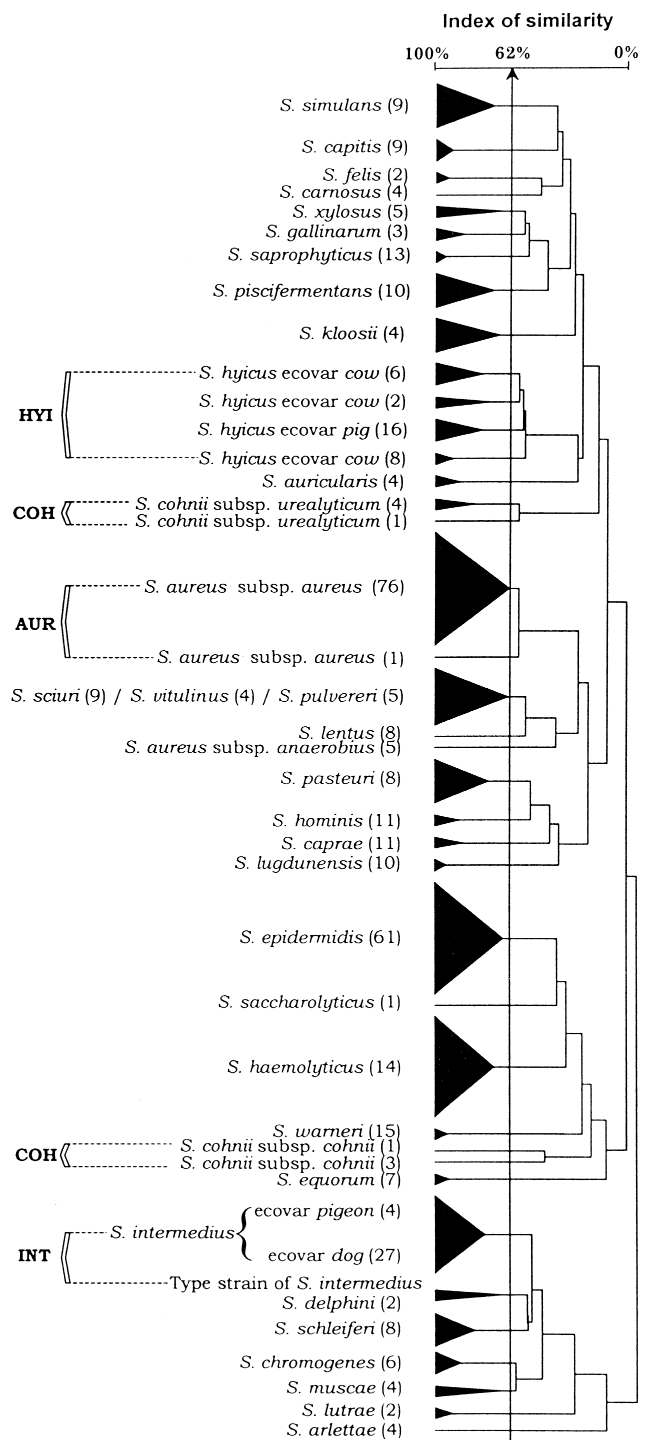

Fig. 2. Dendrogram showing the relatedness of the 120 *Hin*dIII ribotypes included in Staph DB (cluster analysis by the Dice coefficient and UPGMA). The clusters are represented by triangles, the lengths of the bases of which are proportional to the number of ribotypes (the smallest correspond to two ribotypes, e.g. *Staphylococcus felis*). The number of strains tested is given in parentheses. Taxa, strains of which are not clustered, are indicated on the left: AUR, *S. aureus*; COH, *S. cohnii*; HYI, *S. hyicus*; INT, *S. intermedius*.

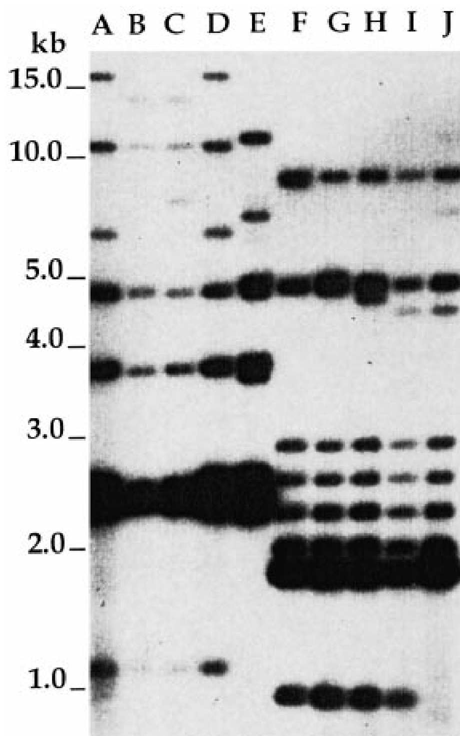

Fig. 3. *Eco*RI ribotypes of 10 *S. intermedius* strains. Lanes A–D, strains from pigeons; lane E, type strain of the species; lanes F–J, strains from dogs.

vidualized. With *Hin*dIII, *Staphylococcus delphini*, *Staphylococcus aureus*, *Staphylococcus cohnii* and *S. sciuri* could not be identified and *S. sciuri* was not distinguishable from *S. vitulinus*–*S. pulvereri* (Fig. 2). Analysis of the *Eco*RI ribotypes allowed ecovar-specific clustering of all the strains belonging to *S. intermedius* ecovar *dog*, *S. hyicus* ecovar *pig* and *S. hyicus* ecovar *cow* (Fig. 1). The similarity among the *Eco*RI ribotypes of *S. intermedius* ecovar *dog* strains was evident visually on the radiograph (Fig. 3). With both enzymes, the *S. intermedius* type strain, referenced as ATCC 29963^{T} and first isolated from pigeon, was divergent from the other *S. intermedius* strains from both pigeon and dog. Its *Hin*dIII ribotype was clustered with those of the two *S. delphini* strains. Its *Eco*RI ribotype was the same as that reported previously in another study (Hesselbarth & Schwarz, 1995) and, therefore, it is unlikely that the strain tested was not the *S. intermedius* type strain.

DISCUSSION

Even though quantitative DNA–DNA hybridization of the whole genome remains the 'gold-standard' method for delineation of bacterial taxa (Wayne *et al*., 1987), several approaches have been proposed for the identification of staphylococcal taxa. These approaches fall into two categories: those based on the detection of species-specific sequences that are not present in the chromosome of all staphylococci (Akatova *et al*., 1992; Chesneau *et al*., 1993a; Martineau *et al*., 1996; Vandenesch *et al*., 1995) and those based on the detection of sequence variations in ubiquitous elements such as rRNA and tRNA operons (Bialkowska-Hobrzanska *et al*., 1990; Chesneau *et al*., 1992; De Buyser *et al*., 1989, 1992; Forsman *et al*., 1997; Gaszewska-Mastalarz *et al*., 1998; Hesselbarth & Schwarz, 1995; Irlinger *et al*., 1997; Kluytmans *et al*., 1998; Maes *et al*., 1997; Mendoza *et al*., 1998; Pennington *et al*., 1991; Thomson-Carter *et al*., 1989; Webster *et al*., 1994; Welsh & McClelland, 1992) or chaperonin-encoding genes (Goh *et al*., 1997). The second of these categories of methods may be useful for classifying staphylococci if appropriate databases exist and are updated continuously as data for new taxa become available. The updating study reported here shows that rRNA gene restriction site polymorphism analysis remains a valid approach for delineating most of the species but not the subspecies described in *Staphylococcus*. We also report findings relevant to current issues in staphylococcal taxonomy: (i) whether *S. pulvereri* and *S. vitulinus* are distinct taxonomic entities, despite their ribotypes falling into a single cluster and (ii) whether *S. intermedius* and *S. hyicus* form homogeneous taxonomic groups, despite the ribotypes of strains within these groups being divergent.

There have been numerous changes in the taxonomy of novobiocin-resistant and oxidase-positive staphylococci in recent years, such as the description of two new species, *S. vitulinus* and *S. pulvereri* (Webster *et al*., 1994; Zakrzewska-Czerwińska *et al*., 1995), and the delineation of three subspecies within *S. sciuri* (Kloos *et al*., 1997). Our study shows that the ribotypes of *S. pulvereri* strains were either similar or identical to those of *S. vitulinus* strains. Both species were described in 1995 but by different laboratories and have therefore not been compared to each other previously by molecular techniques. The phenotypic similarities between *S. vitulinus* and *S. pulvereri* strains do not rule out their assignment to different species; some staphylococcal species differ from each other only by a small number of phenotypic traits (Chesneau *et al*., 1993b; Kloos & Lambe, 1991). However, the observation that their ribotypes fall into the same cluster for each of the two enzymes used suggests strongly that they belong to the same species. It is, nevertheless, possible that they are different subspecies and further analysis is required to resolve this issue. A method based on the amplification of sequences from the gene encoding the chaperonin 60 protein (*cpn60*) was used successfully for the comparative analysis of all the staphylococci tested (Goh *et al*., 1997): the sequences of the *cpn60* genes may vary enough to differentiate each of the two subspecies described within *S. capitis* and *S. cohnii*

or may not, as in the case of the two *S. schleiferi* subspecies. Another powerful technique, DNA macrorestriction of the staphylococcal genome, has shown that the two subspecies of *S. schleiferi* cannot be differentiated, whereas the subspecies defined within *S. aureus* and *S. capitis* can be recognized clearly (George & Kloos, 1994; Pantůček *et al.*, 1996). We are aware of the poor performance of rRNA gene restriction site polymorphism analysis to discriminate staphylococcal subspecies (this study; Mendoza *et al.*, 1998), but the other DNA fingerprinting methods tested to date seem to have limitations and none is as accurate as DNA–DNA hybridization. Thus, it would appear that the only way to determine whether *S. pulvereri* and *S. vitulinus* are indeed separate subspecies is a quantitative overall similarity analysis of their genomes, involving determination of the ΔT_m of their hybrids.

The heterogeneity detected within *S. hyicus* on the basis of ribotype analysis (this study) is consistent with the findings of *cpn60* gene analysis (Goh *et al.*, 1997). Both methods grouped the *S. hyicus* strains according to their origin, thus enabling distinction between ecovars of *S. hyicus*. Cow and pig were the two animal hosts that were investigated. The inability to group the *S. hyicus* strains as a single cluster reflects a divergence that may be large enough to assign the two clusters of strains to distinct taxa. Once again, only DNA–DNA hybridization experiments would yield a decisive answer. Another point of accordance between ribotyping (this study) and *cpn60* gene analysis (Goh *et al.*, 1997) concerns the close relatedness between the *S. intermedius* type strain, referenced as ATCC 29963^T, and *S. delphini* strains. The ribotype of the *S. intermedius* type strain is divergent from the two *Eco*RI ribotype subgroups covering the other members of the species, the division into subgroups being consistent with their origin. We suggest, therefore, that the type strain of *S. intermedius* may not be representative of the species. This suggestion is supported strongly by our findings for the gene encoding the thermonuclease, *nucI*, cloned from a canine strain of *S. intermedius* (Chesneau *et al.*, 1992): the *nucI* gene hybridized with all strains tested except the *S. intermedius* type strain (unpublished results). Various animal hosts carry *S. intermedius* strains transiently, and pigeons are probably the natural reservoir of *S. intermedius* (Hesselbarth & Schwarz, 1995). It may be appropriate to select another *S. intermedius* type strain that better represents the genomic characteristics of a larger number of isolates of the species.

Our investigation illustrates various aspects of the value of rRNA gene restriction site polymorphism analysis for staphylococcal taxonomy. As it can be used to identify most of the species reported to date and to classify strains of diverse origin, ribotype determination may be very helpful for reference centres dealing with staphylococci. Some databases of staphylococcal ribotypes exist, but they need to be improved, either by complete automation of the technique to guarantee reproducibility (Kloos *et al.*, 1997; Webster *et al.*, 1994) or by a robust assessment of the method (this study) that guarantees performance. We hope that the recent development and availability of the Qualicon automated system (RiboPrinter) will result in this method becoming more widely used.

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