Genomic Variability of *Staphylococcus aureus* and the Other Coagulase-Positive *Staphylococcus* Species Estimated by Macrorestriction Analysis Using Pulsed-Field Gel Electrophoresis

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The genomic DNAs of 95 culture collection and hospital *Staphylococcus aureus* subsp. *aureus* strains of various origins, as well as the genomic DNAs of other coagulase-positive *Staphylococcus* species, were cleaved with restriction endonuclease *Sma*I and subjected to pulsed-field gel electrophoresis. The levels of similarity of the *Sma*I restriction patterns of the *S. aureus* subsp. *aureus* strains varied from 30 to 100%, which is considered characteristic of this species; thus, these organisms belonged to the same species restriction group. Within this range of similarity values 13 *S. aureus* intraspecies restriction groups were identified, and each group consisted of strains whose levels of similarity ranged from 65 to 100%. *S. aureus* subsp. *aureus* CCM 885^T (T = type strain) belonged to the major intraspecies restriction group that comprised 39% of the *S. aureus* strains which we studied. The strains of the other coagulase-positive staphylococci, including *Staphylococcus aureus* subsp. *anaerobius*, *Staphylococcus hyicus*, *Staphylococcus intermedius*, *Staphylococcus delphini*, and *Staphylococcus schleiferi* subsp. *coagulans*, clustered with their type strains in separate restriction groups. *S. aureus* subsp. *aureus* exhibited almost no similarity to these species. We found 44-kb *Sma*I fragments in all of the *S. aureus* subsp. *aureus* and *S. aureus* subsp. *anaerobius* strains studied, and these fragments are considered characteristic of the species *S. aureus*. The high level of homology of these fragments was confirmed by the results of DNA hybridization experiments in which we used representatives of individual intraspecies restriction groups. Of the other staphylococci studied, only *Staphylococcus epidermidis* and one strain of *S. hyicus* contained these fragments. However, the levels of homology between these fragments and the fragments of *S. aureus* were found to be very low.

Classification of genomic DNA restriction patterns by pulsed-field gel electrophoresis (PFGE) has been shown to be a useful tool for investigating the source, transmission, and spread of nosocomial infections and particularly for epidemiologic typing and determining the genetic relatedness of methicillin-resistant *Staphylococcus aureus* strains (3, 7, 8, 10, 12, 15, 17, 27–30, 34–36, 40), as well as for differentiating clumping factor-negative oxacillin-resistant strains from clumping factor-positive oxacillin-resistant strains (31). Hesselbarth et al. (13) and Khambaty et al. (19) used PFGE to determine the genome variability of *Staphylococcus intermedius*. The importance of PFGE as a tool for epidemiological analysis of the coagulase-negative organisms *Staphylococcus epidermidis* and/or *Staphylococcus haemolyticus* has been discussed by Linhardt et al. (22), Goering and Duensing (10), and Degener et al. (4). This method has also been shown to be useful for studying chromosomal polymorphism in different *Staphylococcus* species (9, 21, 33).

In this study we focused on the following questions. (i) What was the variability in the restriction fragment patterns of genomic DNAs within *Staphylococcus aureus* subsp. *aureus* as determined by using PFGE and a large sample of strains that had different origins? We tried to express this variability by determining the number of intraspecies restriction groups (ISRGs). An ISRG is a group of strains that belong to the

same species and have similar restriction patterns. (ii) What ISRGs were the predominant *S. aureus* subsp. *aureus* ISRGs? (iii) Were there certain restriction fragments that were specific for *S. aureus* subsp. *aureus* and were found in all strains of this subspecies? (iv) Could the coagulase-positive taxa *S. aureus* subsp. *aureus*, *Staphylococcus aureus* subsp. *anaerobius* (5), *Staphylococcus hyicus* (6), *S. intermedius* (11), *Staphylococcus delphini* (38), and *Staphylococcus schleiferi* subsp. *coagulans* (16) be distinguished on the basis of their restriction fragment patterns?

MATERIALS AND METHODS

***Staphylococcus* strains.** The following strains were obtained from the Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic: *S. aureus* subsp. *aureus* CCM 885^T (T = type strain), CCM 1484, CCM 2287, CCM 2286, CCM 2323, CCM 2353, CCM 2512, CCM 5755, CCM 5756, CCM 5757, CCM 5972, CCM 5973, and CCM 6188; *S. aureus* subsp. *anaerobius* CCM 3823^T, CCM 3824, and CCM 3826; *Staphylococcus auricularis* CCM 3991^T; *Staphylococcus capitis* CCM 2734^T; *Staphylococcus caprae* CCM 3573^T; *Staphylococcus caseolyticus* CCM 3540^T; *Staphylococcus chromogenes* CCM 3387^T; *Staphylococcus cohnii* CCM 2736^T; *S. delphini* CCM 4115^T and CCM 4184; *S. epidermidis* CCM 2124^T and CCM 50; *Staphylococcus equorum* CCM 3832^T; *Staphylococcus felis* CCM 4196^T; *Staphylococcus gallinarum* CCM 3572^T; *S. haemolyticus* CCM 2737^T; *Staphylococcus hominis* CCM 3474^T; *S. hyicus* CCM 2995, CCM 2994, and CCM 2368^T; *Staphylococcus kloosii* CCM 3834^T; *S. intermedius* CCM 5739^T, CCM 2885, and CCM 2618; *Staphylococcus lentus* CCM 3472^T; *Staphylococcus lugdunensis* CCM 4064^T; *Staphylococcus muscae* CCM 4175^T; *Staphylococcus pasteurii* CCM 4389^T; *Staphylococcus piscifermentans* CCM 4345^T; *Staphylococcus saprophyticus* CCM 883^T; *Staphylococcus schleiferi* subsp. *schleiferi* CCM 4070^T; *S. schleiferi* subsp. *coagulans* CCM 4313^T and CCM 4315; *Staphylococcus sciuri* CCM 3473^T; *Staphylococcus simulans* CCM 2705^T; *Staphylococcus warneri* CCM 2730^T; and *Staphylococcus xylosus* CCM 2738^T. *Staphylococcus carnosus* DSM 20501^T was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The *S. aureus* strains whose designations

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begin with PS (PS strains) were obtained from V. Hájek, Faculty of Medicine, Olomouc, Czech Republic. *S. aureus* NCTC 8325-4 was obtained from P. A. Pattee, Iowa State University, Ames. *S. aureus* NCTC 8511 and S26 were obtained from R. Z. Korman (United States). *S. epidermidis* O-47, Tü 3298 (= DSM 3095), and RP62a were obtained from the laboratory of F. Götz. Clinical *S. aureus* whose designations begin with FN and R were obtained from seven hospitals in Brno, Czech Republic, over the 3-year period from 1991 to 1994. The biochemical characteristics of these strains (they produce coagulase, clumping factor, hyaluronidase, and alkaline phosphatase, form acid aerobically from D-mannitol, sucrose, and D-trehalose, produce acetoin from glucose, and reduce nitrates) are characteristics which have been described previously for *S. aureus* (14).

Preparation of DNA for PFGE. DNA was isolated by the method described by Linhardt et al. (22) and Prevost et al. (27), with some modifications. Each *S. aureus* strain was grown in 20 ml of 2× YT medium at 37°C to an optical density at 600 of 0.2 to 0.3. The bacteria were harvested, washed twice in 2 ml of a buffer containing 10 mM Tris-HCl, 10 mM EDTA, 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid], and 1 M NaCl (pH 7.5), and resuspended in 200 μl of the same buffer. The suspension was then immediately mixed with 200 μl of 2% low-melting-point agarose (type VII; Sigma Chemical Co.) in 50 mM Tris-HCl-5 mM EDTA (pH 8.0) and stored at 55°C, and then 20 μl of a 0.5-mg/ml lysostaphin (Sigma Chemical Co.) stock solution was added. After agarose blocks were cast, they were transferred into 1 ml of lysis buffer (6 mM Tris-HCl, 100 mM EDTA, 1 M NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% N-lauroylsarcosine; pH 7.6) supplemented with 500 μg of lysozyme (Sigma Chemical Co.) and 10 μg of RNase and incubated for 3 h at 37°C. The lysis buffer was then replaced with 1 ml of proteolysis buffer (0.25 M EDTA, 20 mM EGTA, 1% N-lauroylsarcosine; pH 9.0) containing 500 μg of proteinase K (Sigma Chemical Co.). Digestion was performed for 12 h at 55°C. The agarose blocks were washed five times with 25 ml of TE buffer and stored at 4°C in TE buffer.

Restriction enzyme digestion and PFGE. *Sma*I (Boehringer Mannheim) and *Sst*II (Sigma Chemical Co.) were used for restriction enzyme cleavage. Cleavage was performed with 8 U of enzyme (for agarose blocks that were 1 by 1 by 5 mm) overnight. PFGE was performed by using a CHEF-DR II system (Bio-Rad Laboratories, Inc.) and 1.2% agarose gels at 14°C in 1× TAE buffer. A constant voltage of 170 V was applied with increasing pulse times of 1 to 65 s over a period of 36 h. To precisely estimate fragment sizes, we used pulse times of 0.1 to 35 and 10 to 90 s for small and large fragments, respectively. Bacteriophage lambda DNA concatemers from c185757 (Promega) and *Hind*III restriction fragments of bacteriophage lambda DNA were used as size markers. The gels were stained for 1 h in ethidium bromide, destained in water, and photographed under UV light (302 nm).

Hybridization experiments. The 44-kb *Sma*I fragment from *S. aureus* CCM 885^T was isolated from a gel after we performed PFGE with low-melting-point agarose (Sigma Chemical Co.) and was labeled by a nonradioactive method (DIG DNA labeling and detection kit; Boehringer Mannheim). *Sma*I-digested DNAs of other strains resolved by PFGE were transferred to nylon membranes (Boehringer Mannheim) by Southern blotting. The membranes were prehybridized and hybridized with the probe at 68°C, and the bands were detected as recommended by the supplier.

Estimating levels of restriction pattern similarity. The restriction patterns of DNAs isolated from different strains were compared quantitatively by using the formula proposed by Upholt (37) and Nei and Li (24), $F_{xy} = 2n_{xy}/(n_x + n_y)$, where F_{xy} is the proportion of restriction fragments that are found in both of the strains compared; n_x and n_y are the total numbers of restriction fragments found in strains x and y , respectively; and n_{xy} is the number of restriction fragments found in both strain x and strain y . The gels were evaluated visually. Only patterns on the same gel were compared. F_{xy} values were estimated by performing three independent experiments, and nonequimolar fragments (including partially digested fragments) were not considered when we estimated levels of restriction pattern similarity. Distance matrices of F_{xy} values were subjected to a cluster analysis in order to estimate the levels of restriction pattern similarity for strains as described by Snopková et al. (33). The unweighted pair group average linkage method was used to construct dendrograms.

RESULTS

In order to estimate the proper levels of similarity of *Sma*I restriction patterns for forming ISRGs, we subjected 23 *S. aureus* PS strains to PFGE and compared their restriction patterns. We chose these strains because (i) they had different origins; (ii) Pattee et al. (26) and Rosdahl et al. (28) found that strains sensitive to phages belonging to the same lytic group produced similar restriction patterns; and (iii) they are well known internationally because they are used as the standard hosts for the phages belonging to the International Standard Set for phage typing. On the basis of their sensitivity to the International Standard Set of phages for phage typing, the PS strains were divided into five phage lytic groups (2, 23). The

*Sma*I restriction patterns of these organisms are shown in Fig. 1A. The dendrogram in Fig. 2A shows that most of the PS strains that were sensitive to the phages belonging to the same lytic group were members of the same cluster at levels of similarity ranging from 65 to 100%. Clusters were formed by the strains that were sensitive to the phages belonging to lytic groups I, II, III, and V. Only strains PS 3A and PS 42E (members of lytic groups II and III, respectively) were not placed in their appropriate clusters; the levels of similarity between these strains and the members of the clusters were less than 65% similarity. Strains PS 95 and PS 187, which belonged to miscellaneous lytic group M, formed separate clusters, and PS 81 strain, a member of the same lytic group, clustered with the strains belonging to lytic group I, which is consistent with the results of Asheshov and Winkler (1), who reported that this organism was related to group I.

Our results indicated that the PS strains belonging to the same lytic group were genetically closely related since the levels of similarity of their restriction patterns ranged from 65 to 100%. However, this does not mean that these organisms are related only on the basis of sensitivity to certain bacteriophages. It does mean that their genomes are similar (related) since *Sma*I restriction sites are distributed along the whole *S. aureus* chromosome, as shown by the restriction map constructed by Pattee (25). Since other authors (see below) also obtained levels of relatedness ranging from 65 to 100% for the strains which they studied, we used this range to estimate the boundaries of the ISRGs. Using these boundaries, we identified 13 ISRGs among 95 *S. aureus* strains that had different origins. The characteristics of these ISRGs are shown in Table 1. The 95 strains studied produced 78 different *Sma*I restriction patterns.

In order to make it possible to place unknown strains into their appropriate ISRGs (see below), one strain of each group was chosen as its representative. The *Sma*I restriction patterns of these representative strains are shown in Fig. 1B. The dendrogram in Fig. 2B shows that the interstrain levels of similarity were less than 65% but not less than 30% when these strains were examined.

The ISRGs differed from one another in the numbers of strains that they included (Table 1). ISRG 1 was the largest ISRG and contained 39% of all of the *S. aureus* strains studied. This group included well-defined culture collection strains, as well as PS strains sensitive to the phages belonging to lytic group III. The PS strains which were hosts for the phages belonging to lytic group I were members of ISRG 6, which contained 14% of the strains studied. Each of the other ISRGs contained less than 10% of the 95 strains studied.

As shown in Fig. 3, some restriction fragments seemed to characterize restriction groups being shared by all of the strains of an ISRG. In addition, some fragments occurred in several ISRGs; for example, a 134-kb fragment was found in members of ISRGs 2, 5, and 7 through 9, a 116-kb fragment was found in members of ISRGs 1 and 3 through 10, a 76-kb fragment was found in members of ISRGs 4, 5, and 8 through 11, a 23-kb fragment was found in members of ISRGs 1 through 6, 8, and 9, a 19-kb fragment was found in members of ISRGs 1 through 5, 9, and 10, and a 9.8-kb fragment was found in members of ISRGs 1 through 9.

*Sma*I restriction fragments that were 44 ± 1.5 kb long were found in all of the *S. aureus* strains which we studied.

We also studied whether the restriction patterns of the *S. aureus* subsp. *aureus* strains were similar to the restriction patterns of the other coagulase-positive *Staphylococcus* species. In such a study clustering the strains of different species requires the use of the type strains of the species studied; the

FIG. 1. (A) PFGE of *SmaI*-cleaved genomic DNAs of *S. aureus* strains used as hosts for the phages belonging to the International Standard Set. Lane L, molecular size marker (lambda concatemers). Lane 1, PS 29; lane 2, PS 52; lane 3, PS 52A; lane 4, PS 80; lane 5, PS 3A; lane 6, PS 3C; lane 7, PS 55; lane 8, PS 71; lane 9, PS 6; lane 10, PS 42E; lane 11, PS 47; lane 12, PS 53; lane 13, PS 75; lane 14, PS 54; lane 15, PS 77; lane 16, PS 83A; lane 17, PS 84; lane 18, PS 85; lane 19, PS 81; lane 20, PS 95; lane 21, PS 94; lane 22, PS 96; lane 23, PS 187. (B) PFGE of *SmaI*-cleaved genomic DNAs of representative strains of the *S. aureus* ISRGs listed in Table 1. Lane 1, CCM 885^T; lane 2, PS 85; lane 3, FN 15; lane 4, PS 3A; lane 5, CCM 2323; lane 6, PS 80; lane 7, PS 96; lane 8, CCM 2287; lane 9, PS 55; lane 10, PS 95; lane 11, PS 187; lane 12, FN 4; lane 13, PS 42E; lane 14, *S. aureus* subsp. *anaerobius* CCM 3823^T; lane L, molecular size marker (lambda concatemers). The estimated sizes of *S. aureus* CCM 885^T *SmaI* fragments are indicated on the left.

strains belonging to the same species should cluster with the type strain, forming a species restriction group. The range of levels of similarity of the restriction fragment patterns of members of a species restriction group depends on the genomic variability of the strains belonging to the species (33). This study was performed by using two restriction endonucleases, *SmaI* (Fig. 4) and *SstII* (data not shown). As Fig. 5 shows, the

FIG. 2. (A) Dendrogram showing the levels of similarity of the *SmaI* restriction patterns of the genomic DNAs of *S. aureus* strains used as hosts for the phages belonging to the International Standard Set for phage typing. The phage lytic groups to which the PS strains belong are indicated at the top. *S. aureus* CCM 885^T was included for comparison. (B) Dendrogram showing the levels of similarity of the *SmaI* restriction patterns of the genomic DNAs of *S. aureus* strains that were used as representatives of ISRGs. Strain PS 187 has not belonged to the International Standard Set for phage typing since 1974 (2).

dendrograms showing the levels of similarity of the *SmaI* and *SstII* restriction patterns of the genomic DNAs of the coagulase-positive *Staphylococcus* strains are very similar. Individual coagulase-positive strains clustered together with their appropriate type strains in separate species restriction groups. The same clusters were obtained with both *SmaI* and *SstII* restriction endonucleases. The range of levels of similarity was 21 to 100% when *SmaI* was used, and the range of levels of similarity was 30 to 100% when *SstII* was used. The coagulase-negative organism *S. schleiferi* subsp. *schleiferi* CCM 4070^T clustered with the coagulase-positive strains of *S. schleiferi* subsp. *coagulans*. Both of the dendrograms also showed that the levels of similarity between the two subspecies of *S. aureus* and the other coagulase-positive species were very low (about 3%). The level of similarity between *S. aureus* subsp. *anaerobius* and the type strain *S. aureus* subsp. *aureus* CCM 885^T was only 17%.

We estimated the sizes of the restriction fragments of *S. aureus* CCM 885^T. Figure 1 shows that the restriction fragment sizes ranged from 9.8 to 621 kb. The sizes of 13 *SmaI* fragments corresponded to the sizes of fragments C, D, G, H, I, J, K, L, and M and four small fragments whose sizes in *S. aureus* NCTC 8325 had been estimated previously by Pattee (25) and Pattee et al. (26). The sizes of the restriction fragments in whole samples of the strains which we studied ranged from 7.5 to approximately 1,200 kb. The molecular size of the genome of *S. aureus* CCM 885^T was estimated to be $2,766 \pm 45$ kb. The average molecular sizes of the genomes of 95 *S. aureus* subsp. *aureus* strains ranged from 2,707 to 3,073 kb. The genome sizes of coagulase-positive species differed from one another (Table 2).

As stated above, a 44-kb *SmaI* fragment was found in all of the *S. aureus* strains which we studied. This fragment corresponds to fragment L on the genome map of *S. aureus* NCTC

TABLE 1. Characterization of *S. aureus* subsp. *aureus* ISRGs

Group	Representative strain	No. of strains	Designations of strains as received	No. of <i>Sma</i> I restriction fragments	Range of F_{xy} values
ISRG 1	CCM 885 ^T	37	CCM 885 ^T , CCM 1484, CCM 5755, CCM 5756, CCM 5757, CCM 5973, CCM 6188, NCTC 8511, NCTC 8325-4, PS 47, PS 53, PS 77, PS 75, PS 54, PS 83A, PS 84, S 26, FN 12, FN 29, FN 34, FN 42, FN 43, FN 45, FN 46, FN 51, FN 63, FN 73, FN 74, FN 81, FN 93, R 8, R 171, R 232, R 176, R 191, R 235, R 310	17-20	0.68-1.00
ISRG 2	PS 85	4	PS 85, PS 6, CCM 5972, FN 22	18-21	0.65-0.75
ISRG 3	FN 15	6	FN 15, FN 8, FN 25, R 120, R 152, R 206	18	0.75-1.00
ISRG 4	PS 3A	9	PS 3A, FN 36, R 26, R 30, R 117, R 119, R 138, R 665, R 787	18-20	0.70-0.98
ISRG 5	CCM 2323	1	CCM 2323	19	1.00
ISRG 6	PS 80	13	PS 80, PS 81, PS 29, PS 52, PS 52A, SA 812, FN 67, FN 76, R 36, R 58, R 146, R 153, R 198	17-21	0.70-0.98
ISRG 7	PS 55	5	PS 55, PS 3C, PS 71, CCM 2353, R 5	16-17	0.65-0.74
ISRG 8	PS 96	5	PS 96, PS 94, R 98, R 124, R 309	19	0.76-1.00
ISRG 9	CCM 2287	2	CCM 2287, CCM 2286	17-18	0.90
ISRG 10	PS 42E	2	PS 42E, R 194	14-16	0.73
ISRG 11	PS 95	5	PS 95, CCM 2512, R 84, R 44, R 104	16-20	0.70-1.00
ISRG 12	PS 187	2	PS 187, R 826	18	0.87
ISRG 13	FN 4	4	FN 4, FN 21, R 49, R 240	13	0.77-1.00

8325 (25). In addition, we determined whether this fragment also occurred in the type strains of 25 coagulase-negative *Staphylococcus* species. We found it only in *S. epidermidis* CCM 2124^T. Because of this, other *S. epidermidis* strains (strains CCM 50, O-47, Tü 3298, and RP62a) were also studied and were found to possess this fragment. Only one coagulase-positive non-*S. aureus* strain, *S. hyicus* CCM 2995, was found to have this fragment.

We also tried to determine whether the 44-kb fragments of the different strains were homologous. To do this, the 44-kb fragment was isolated from *S. aureus* CCM 885^T and used as a probe for hybridization in Southern blots of the restriction patterns of representative strains. Figure 6 shows that the 44-kb fragments of all of the strains representing *S. aureus* subsp. *aureus* and *S. aureus* subsp. *anaerobius* ISRGs were

homologous. The levels of homology with the 44-kb fragments of all of the *S. epidermidis* strains studied and *S. hyicus* CCM 2995 were low.

DISCUSSION

The 13 ISRGs described in this paper indicate that there is considerable genomic variability in *S. aureus*. Whether the ISRGs identified by using the strains which we studied represent all of the *S. aureus* subsp. *aureus* ISRGs or whether more ISRGs could still be found is not known. The range of similarity values (65 to 100%) used to define the boundaries of ISRGs based on *Sma*I restriction patterns was substantiated not only by the results described in this paper but also by the results described by other authors. The clusters of PS strains

FIG. 3. Diagram showing the *Sma*I restriction fragments found in all strains of *S. aureus* ISRGs 1 to 13. The sizes of individual fragments are indicated on the left.

FIG. 4. PFGE of *Sma*I-cleaved genomic DNAs of coagulase-positive species. Lane 1, CCM 885^T; lane 2, CCM 3823^T; lane 3, CCM 3824; lane 4, CCM 3826; lane 5, CCM 2368^T; lane 6, CCM 2994; lane 7, CCM 2995; lane 8, CCM 2618; lane 9, CCM 2885; lane 10, CCM 5739^T; lane 11, CCM 4115^T; lane 12, CCM 4184; lane 13, CCM 4313^T; lane 14, CCM 4315; lane 15, CCM 4070^T; lane L, molecular size marker (λ concatamers).

obtained by using this range of similarity values are almost completely consistent with the clusters described by Rosdahl et al. (28). Only the position of strain PS 3A is questionable; this strain was placed in different clusters in the two studies.

Struelens et al. (35, 36) used coefficients of similarity calculated by using *Sst*II and/or *Sma*I macrorestriction PFGE patterns to analyze the DNA relatedness of methicillin-resistant *S. aureus* strains from different sources. These authors defined major macrorestriction genotypes (or clones) on the basis of common electrophoretic restriction patterns that differed by three or fewer fragments and exhibited similarity coefficients greater than 85%, a value which is consistent with the range of similarity values estimated for our ISRGs.

Thus, the placement of closely related strains by Rosdahl et al. (28) and Struelens et al. (35, 36) by using similarity values greater than 65% supports the range which we suggest for formation of ISRGs. We were not able to compare our data with the data which were used by El-Adhami et al. (8) and Stewart et al. (34) to construct dendrograms because the dendrograms of these authors were based on *p* values, which expressed the estimated fractions of nucleotide differences in pairs of DNA samples.

We emphasize that our cluster analysis (Table 1) was performed with intention of forming as many ISRGs as possible in order to determine the variability of *S. aureus* subsp. *aureus* genomes. For this reason a large number of strains of different origins were included in this analysis. The results of our anal-

FIG. 5. Dendrograms showing the levels of similarity of the *Sma*I (A) and *Sst*II (B) restriction patterns of the genomic DNAs of different coagulase-positive species. The coagulase-negative organism *S. schleiferi* subsp. *schleiferi* CCM 4070^T was included for comparison.

ysis are fully reproducible if samples of the same size and composition are used. If the size and composition of the samples are changed, some differences at the threshold ISRG similarity values may occur. This follows from the data shown in Table 1 and Fig. 2A. On Fig. 2A *S. aureus* CCM 885^T and PS 85 occur in the same cluster (range of levels of similarity, 65 to 100%), whereas these strains were members of different ISRGs (ISRG 1 and ISRG 2) (Table 1). This was because the level of similarity of these strains was on the threshold ($F_{xy} = 0.67$). On the other hand, despite the great difference in the sizes of the samples used in the cluster analyses whose results are shown in Table 1 and Fig. 2A, the compositions of the other clusters correspond to the compositions of the ISRGs shown in Table 1.

The levels of similarity between strains that were representatives of different ISRGs (Table 1) were less than 65%. This is shown in Fig. 2B, in which the highest level of similarity is 60% (the level of similarity between strains CCM 885^T and PS 85) and the lowest level of similarity is 30% (the level of similarity between strain PS 42E and the other strains). Therefore, the representative strains can be used as reference (standard) strains to place unknown strains into the 13 ISRGs. To do this, the F_{xy} value for the unknown strain and each of the representative strains of the ISRGs has to be determined. The unknown strain is placed into the ISRG, with whose representative strain exhibits the highest F_{xy} value. This F_{xy} value must be more than 65%.

Shifts in restriction patterns may be due to mutations in restriction sites, as well as to the presence of prophages and other heterologous insertions of DNA in the chromosome of *S. aureus* (26). According to Smeltzer et al. (32), all of the *Sma*I-defined restriction fragment length polymorphisms observed in *S. aureus* NCTC 8325, NCTC 8325-4, KSI1201, KSI1202, S6C, and PS 42D were the direct result of the presence or absence of prophage DNA in the staphylococcal genome. Lina et al. (20) found that chromosomal integration of phage *118II* could

TABLE 2. Basic characterization of the coagulase-positive *Staphylococcus* species which we studied

Coagulase-positive species or subspecies	Strain ^a	<i>Sma</i> I digestion		<i>Sst</i> II digestion	
		No. of restriction fragments	Molecular size of genome (kb) ^b	No. of restriction fragments	Molecular size of genome (kb)
<i>S. aureus</i> subsp. <i>aureus</i>	CCM 885 ^T	17	2,766 ± 45	ND ^c	ND
<i>S. aureus</i> subsp. <i>anaerobius</i>	CCM 3823 ^T	17	3,160 ± 85	16	3,010 ± 129
	CCM 3824	17	3,177 ± 95	16	3,061 ± 137
	CCM 3826	18	3,202 ± 65	16	3,073 ± 98
<i>S. hyicus</i>	CCM 2995	25	2,106 ± 109	30	2,170 ± 75
	CCM 2994	28	2,026 ± 83	32	2,104 ± 105
	CCM 2368 ^T	28	2,155 ± 90	35	2,249 ± 81
<i>S. intermedius</i>	CCM 5739 ^T	31	2,428 ± 84	35	2,478 ± 75
	CCM 2885	27	2,155 ± 106	37	2,086 ± 92
	CCM 2618	23	2,393 ± 114	34	2,481 ± 119
<i>S. schleiferi</i> subsp. <i>coagulans</i>	CCM 4313 ^T	33	1,812 ± 78	32	2,049 ± 85
	CCM 4315	27	1,583 ± 75	28	1,708 ± 79
<i>S. delphini</i>	CCM 4115 ^T	25	2,432 ± 94	37	2,537 ± 112
	CCM 4184	25	2,432 ± 94	37	2,537 ± 112

^a Designations as received.^b The molecular size of the strain CCM 885^T genome was determined from measurements obtained from 10 gels; the molecular sizes of the genomes of the other strains were determined from measurements obtained from 3 gels.^c ND, not done.

be responsible for minor stable variations in DNA restriction patterns in coagulase-negative staphylococci.

Do the ISRGs described in this paper apply only to the strains which we studied, or do they have general validity for the species *S. aureus*? The following facts support the latter possibility: (i) the set of strains which we studied included both hospital and culture collection strains having different origins; (ii) the PS strains used as hosts for the phages belonging to the International Standard Set, as well as the culture collection strains, were represented in all of the ISRGs; and (iii) strain CCM 885^T, as well as strains NCTC 8325-4, NCTC 8325, and NCTC 8511, which were used in genetic studies, were members of ISRG 1, which was the largest ISRG.

When we examined the results of our restriction fragment analysis of coagulase-positive strains, we concluded that the classification of these organisms into species restriction groups on the basis of the results of a cluster analysis of the levels of

similarity of the restriction fragment patterns corresponded to their classification into separate species (5, 6, 11, 16, 38). Strains of the coagulase-positive organism *S. schleiferi* subsp. *coagulans* clustered with the coagulase-negative organism *S. schleiferi* subsp. *schleiferi* CCM 4070^T, which is also consistent with the DNA-DNA relatedness results described by Igimi et al. (16).

The 44-kb *Sma*I fragments probably characterize the species *S. aureus*. Therefore, we suggest that in combination with other characteristics (production of coagulase, acetoin, and hyaluronidase and mannitol fermentation) the presence of a 44-kb fragment in the restriction fragment patterns of *Staphylococcus* strains could be used to identify organisms as *S. aureus* strains, as well as to differentiate this species from other *Staphylococcus* species. This suggestion is not weakened by the occurrence of a 44-kb fragment in *S. epidermidis* and *S. hyicus* strains. The 44-kb fragments of the latter species exhibit only low levels of homology with the 44-kb fragments of *S. aureus* strains (and the *S. aureus* CCM 885^T probe). The same is true of *S. caseolyticus* CCM 3540^T, *S. hominis* CCM 3474^T, *S. warneri* CCM 2730^T, *S. pasteurii* CCM 4390^T, and *S. xylosus* CCM 2738^T, in which we found 42-kb fragments (Fig. 6) that exhibited only low levels of homology with the 44-kb fragment.

Carles-Nurit et al. (3) detected 44-kb *Sma*I fragments in both methicillin-resistant and methicillin-sensitive *S. aureus* strains and found that these fragments were identical. As stated above, the 44-kb fragment corresponds to *Sma*I fragment L. In *S. aureus* NCTC 8325 one of the gene markers (Ω1119, a silent insertion of tetracycline resistance transposon Tn916) has been localized in this fragment (18, 25). Transposon Tn916 also occurs in *Sma*I fragments A, D, E, H, I, and M (25) on the physical and genetic map of *S. aureus* NCTC 8325. Wada et al. (39) localized six rRNA operons on the chromosome of *S. aureus* BB225, a derivative of strain NCTC 8325. Two of these operons were located at the junctions of *Sma*I fragment L and neighboring fragments, and the others were located at the junctions of fragments H, I, F, and K and fragment D. In *S. aureus* CCM 885^T our 44-kb fragment probe hybridized weakly with fragments, whose sizes corresponded to the sizes of labelled fragments A and D or fragments E, H, and I and to a 23-kb fragment on which the genes coding for rRNA

FIG. 6. DNAs digested with *Sma*I were subjected to PFGE, transferred to a nylon membrane, and hybridized with the *Sma*I 44-kb probe. Lanes 1 through 13, *S. aureus* subsp. *aureus* CCM 885^T, PS 85, FN 15, PS 3A, CCM 2323, PS 80, PS 96, CCM 2287, PS 55, PS 95, PS 187, FN 4, and PS 42E, respectively; lane 14, *S. aureus* subsp. *anaerobius* CCM 3823^T; lanes 15 and 16, *S. hyicus* CCM 2368^T and CCM 2995, respectively; lane 17, *S. intermedius* CCM 5739^T; lane 18, *S. delphini* CCM 4115^T; lane 19, *S. schleiferi* subsp. *coagulans* CCM 4313^T; lanes 20 through 24, *S. epidermidis* CCM 2124^T, CCM 50, O-47, Tü 3298, and RP62, respectively; lane 25, *S. hominis* CCM 3474^T; lane 26, *S. warneri* CCM 2730^T; lane 27, *S. pasteurii* CCM 4390^T; lane 28, *S. xylosus* CCM 2738^T.

and Tn916 were present. As the 44-kb fragment probe hybridized strongly with the 44-kb fragments of different *S. aureus* strains and only weakly with fragments A and D or fragments E, H, and I and the 23-kb fragment, we assumed that there is another sequence on this fragment, which is conservative since it occurs in all of the ISRGs.

The average molecular size of the genomic DNA of *S. aureus* was estimated to be $2,878 \pm 20$ kb, which is consistent with the results obtained by Prevost et al. (27). However, in contrast to these authors, we did not find a genome molecular size less than 2,700 kb in the strains which we studied.

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