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Overlapping Population Structures of Nasal Isolates of *Staphylococcus aureus* from Healthy Dutch and American Individuals▽

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To understand *Staphylococcus aureus* nasal carriage and its relationship with subsequent disease, insight into the natural (nonclinical) bacterial population structure is essential. This study investigated whether the distributions of *S. aureus* genotypes that cause colonization differ by geographic locales. High-throughput amplified fragment length polymorphism (AFLP) analysis was performed on nasal isolates of *S. aureus* from healthy American ($n = 391$) and Dutch ($n = 829$) volunteers. In total, 164,970 binary outcomes, covering 135 different markers per isolate, were scored. Methicillin resistance was defined for all strains; pulsed-field gel electrophoresis typing was performed for the American isolates. The overall population structures of the American and Dutch *S. aureus* isolates were comparable. The same four major AFLP clusters (I to IV) and subclusters were identified for both collections. However, the Dutch methicillin-susceptible *S. aureus* (MSSA) isolates were overrepresented in AFLP cluster III ($P = 0.0016$). Furthermore, the majority of the American methicillin-resistant *S. aureus* isolates (90.5%) were located in AFLP cluster I ($P < 0.0001$). This result identifies differences in the local prevalence of certain *S. aureus* genotypes. AFLP clusters II and III, which represent multilocus sequence typing clonal complexes 30 and 45, respectively, account for 46.4% of all MSSA isolates in the study, suggesting that these two lineages have evolved as extremely successful pandemic colonizers of humans. In conclusion, the overall population structures of American and Dutch nasal carriage isolates of *S. aureus* are surprisingly similar, despite subtle geographic differences in the prevalence of certain *S. aureus* genotypes.

Since many *Staphylococcus aureus* infections occur in persons with prior bacterial colonization of the anterior nares, knowledge of the human nasal colonization state is of clinical importance (12). In addition, since some strains of *S. aureus* appear to have a higher potential to cause disease than others, it is essential that studies of nasal carriage include bacterial strain typing information (6, 12). Studies of *S. aureus* nasal carriage have been performed in various geographic regions, including the United States (3) and The Netherlands (14), and some of these studies included epidemiological typing of the bacterial isolates obtained (3, 6). However, whether the distributions of *S. aureus* genotypes from various geographical locales differ significantly or are rather similar remains unclear. We here describe the first high-throughput genotyping effort for large numbers of nonclinical isolates of *S. aureus* from healthy volunteers living in the United States and The Netherlands.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains from the United States that were included in this study were collected from civilian, noninstitutionalized U.S. citizens during the National Health and Nutrition Examination Survey in 2001 to 2002. In total, nasal samples from 9,622 persons (≥1 year old) were obtained as previously described (3). *S. aureus* was isolated from 2,964 individuals over the 2-year period. Every 10th methicillin-susceptible *S. aureus* (MSSA) isolate ($n = 307$) and all methicillin-resistant *S. aureus* (MRSA) isolates ($n = 84$) were included in the study. Nine additional MRSA isolates that were excluded from the study by Kuehnert et al. (3) were included in the present study (these isolates were excluded earlier because they were obtained from persons outside the target cohort). Strain characteristics of the American isolates are presented in Table 1. Forty-two isolates demonstrated pulsed-field gel electrophoresis (PFGE) patterns that did not fit within established U.S. lineages (5).

The Dutch strains ($n = 829$) were collected during two different carriage surveys among children and the elderly. In total, 3,198 children (aged 1 to 19 years) from the city of Rotterdam that participated in a national meningococcal vaccination campaign (in 2002) were enrolled. *S. aureus* was isolated from 1,116 of these children (1). All isolates were stored at −80°C in broth containing glycerol. A random sample of 400 *S. aureus* carriage isolates was drawn for this cohort (6). The second collection originated from a community-based prospective study of the elderly in Rotterdam (2). Nasal swabs were obtained from 3,851 persons over 55 years of age, between 1 April 1997 and 31 December 1999. *S. aureus* was isolated from 1,043 elderly persons (9). All isolates were stored at −80°C in glycerol containing broth. A random sample of 429 carriage isolates was drawn (6). The genotyping data and demographic characteristics for the 829 Dutch strains have been described previously (6). All Dutch isolates were MSSA; the absence of *mecA* was confirmed by PCR assay (7). Two *S. aureus* reference strains, N315 and Mu50, both of which have been completely sequenced, were included (4). In total, 1,222 *S. aureus* strains were typed by amplified fragment length polymorphism (AFLP).

TABLE 1. Microbiological characteristics of the 391 American *S. aureus* isolates

PFGE type	Total no. (% of total) of isolates	Total no. (% of PFGE type) of isolates that were:	
		MSSA	MRSA
USA100	46 (11.8)	2 (4.3)	44 (95.7)
USA200	89 (22.8)	88 (98.9)	1 (1.1)
USA300	20 (5.1)	13 (65.0)	7 (35.0)
USA400	6 (1.5)	5 (83.3)	1 (16.7)
USA500	4 (1.0)	3 (75.0)	1 (25.0)
USA600	39 (10.0)	38 (97.4)	1 (2.6)
USA700	13 (3.3)	7 (53.8)	6 (46.1)
USA800	38 (9.7)	21 (55.3)	17 (44.7)
USA900	28 (7.2)	28 (100)	0
USA1000	10 (2.6)	7 (70.0)	3 (30.0)
USA1200	6 (1.5)	6 (100)	0
Group A	18 (4.6)	18 (100)	0
Group B	10 (2.6)	10 (100)	0
Group C	6 (1.5)	6 (100)	0
Group D	11 (2.8)	11 (100)	0
Group E	4 (1.0)	4 (100)	0
Iberian	1 (0.3)	0	1 (100)
Unique	42 (11.0)	40 (93.0)	2 (4.7)
Total	391 (100)	307 (78.3)	84 (21.4)

AFLP. AFLP analysis was performed on purified DNA (MagNA Pure LC instrument; Roche Diagnostics, Almere, The Netherlands) from all 1,222 *S. aureus* strains as described by Melles et al. (6). We used the predictive software package Recomb (10) to select optimal enzyme and primer combinations (Keygene N.V., U.S. patent application WO 00/44937). DNA was digested with MboI and Csp6I, and the linker oligonucleotide pairs for MboI (5′-CTCGTAGACTGCGTACC-3′ and 5′-GATCGGTACGCAGTCTAC-3′) and Csp6I (5′-GACGATGAGTCCTGAC-3′ and 5′-TAGTCAGGACTCAT-3′) were ligated. Subsequently, nonselective preamplification was performed. In the final amplification, a labeled MboI primer containing a single selective nucleotide (either C or G) and a Csp6I primer containing two selective nucleotides (TA) were used. Amplified material was analyzed using polyacrylamide slab gels and autoradiography. Marker fragments were scored and a binary table of fragment absence (indicated by "0") or presence (indicated by "1") was constructed. AFLP technology is covered by patent applications owned by Keygene N.V. (2a, 2b).

AFLP data analysis. Analysis of the AFLP data was performed as described previously (6). The method used for two-dimensional clustering of the data was agglomerative (successive) hierarchical. This clustering was performed using the unweighted-pair group method with arithmetic mean, and the similarity metric was Tanimoto. This defines similarity for binary data (0 and 1) based on the number of positive attributes that two records have in common. The resulting dendrogram (Fig. 1) was ordered by average value.

Principle-component analysis (PCA) is a standard multivariate method to reduce the multidimensional space of the data to its principle component (PC). The PC computation is displayed as a three-dimensional scatter plot in which the position along the axes shows the PCA score of the strain. PCA was used to identify subgroups of AFLP clusters as hidden by a two-dimensional representation of hierarchical clustering. The distribution of the strains in four branches was defined on the basis of PCA. Both hierarchical cluster analysis and PCA were performed using Spotfire DecisionSite 7.2 software. To compare the distributions of strain categories in different phylogenetic lineages, Fisher's exact test was used. A two-sided P value of less than 0.05 was considered significant.

Other laboratory methods. The 391 American *S. aureus* isolates were screened for oxacillin resistance by using the Clinical and Laboratory Standards Institute disk diffusion method as previously described (8). PFGE was performed for the 391 American *S. aureus* isolates by using SmaI. The PFGE patterns were analyzed using BioNumerics (Applied Maths), and isolates were grouped into PFGE clonal types by using Dice coefficients and a value of >80% relatedness (3, 5).

RESULTS

Using the set of 1,222 *S. aureus* isolates, a total of 164,970 binary outcomes were generated, covering 135 scorable AFLP marker fragments per isolate. The AFLP data for the 829 Dutch isolates, the 391 American isolates, and the two reference strains (Mu50 and N315) were subjected to two-dimensional hierarchical cluster analysis of which the graphical output is shown in Fig. 1. This figure indicates the presence of the American isolates and clearly shows that the overall population structures of the American and Dutch isolates are rather comparable: the grouping of the strains into four major AFLP clusters as defined before for the Dutch isolates remains valid (Fig. 2) (6). The American isolates do not segregate from the Dutch isolates, and no additional major *S. aureus* clones were identified. Most importantly, strains of homogeneous clusters II (clonal complex 30 [CC30] by multilocus sequence typing) and III (CC45) seem to be important "*S. aureus* carriage lineages" in both The Netherlands and the United States. However, there are some differences in the distribution of the Dutch and American isolates across the major AFLP clusters (Table 2). A greater proportion of isolates of the Dutch collection than of the U.S. collection of strains belonged to AFLP cluster III ($P = 0.0016$). Additionally, all 829 Dutch strains were methicillin susceptible, whereas in the original U.S. collection, 75 of 2,964 (2.5%) isolates were resistant to methicillin ($P < 0.0001$), with a weighted prevalence of MRSA colonization among the U.S. population of 0.8% (75 of 9,622) MRSA isolates in the U.S. cohort (3). Table 2 shows that the majority of the (American) MRSA isolates (90.5%) are located in AFLP cluster I, which is a genetically heterogeneous cluster (overrepresentation of MRSA isolates in cluster I compared to the other AFLP clusters; $P < 0.0001$). Furthermore, Fig. 3 shows that most MRSA strains in AFLP genetic lineage I are clustered separately in a small subcluster named Ia (embracing two PFGE lineages, USA100 and USA800, as shown in Fig. 4). Subcluster Ia embraces 24 American MSSA isolates (7.8% of all American MSSA strains included in this study), while it embraces 60 American MRSA isolates (71.4% of all American MRSA strains included in this study; $P < 0.0001$).

Figure 4 shows the comparison of AFLP and PFGE typing of the American *S. aureus* carriage isolates. PFGE typing confirms the genetic homogeneity in AFLP clusters II and III as well as the relatively high genetic heterogeneity in AFLP clusters I and IV. The different PFGE clonal types (e.g., USA700, USA900, group A, and group B,) in AFLP cluster I are clustered separately, indicating the similarity between the two typing methods. Furthermore, as shown in Fig. 4, AFLP analysis still identifies genetic heterogeneity among several clonal PFGE types, indicating its high discriminatory power. As described earlier, multilocus sequence typing (MLST) analyses of strains assigned to AFLP clusters I and IV also identified different clonal lineages (including CC5, CC8, CC15, CC20, and CC25 in AFLP cluster I and CC22 and CC121 in cluster IV) (6). In contrast, AFLP clusters II and III are genetically homogeneous lineages that have previously been corroborated by MLST and now are confirmed by PFGE analysis. Strains in AFLP cluster II all belong to CC30 (and PFGE type USA200), and strains in AFLP cluster III all belong to CC45 (and PFGE type USA600).

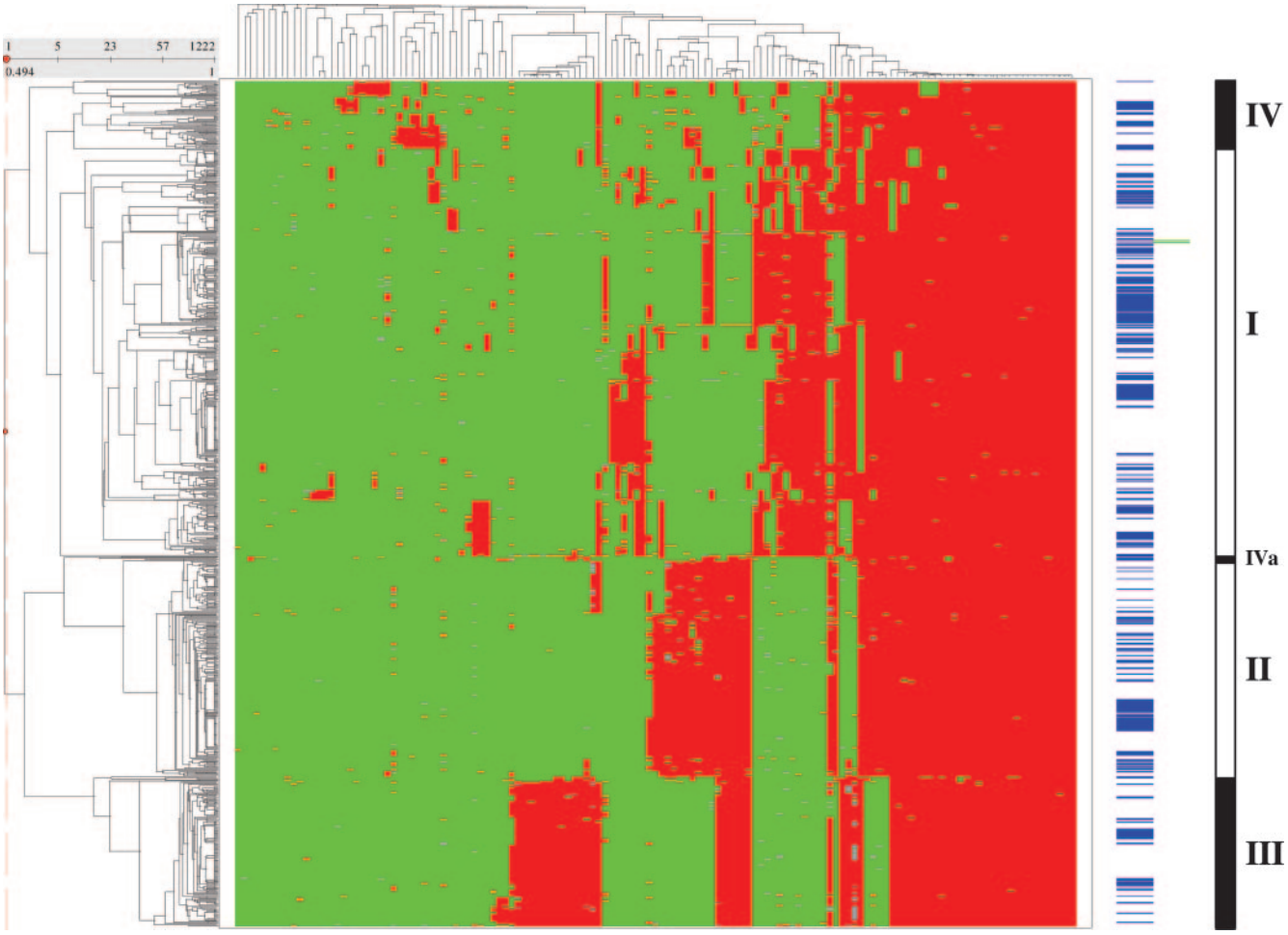

FIG. 1. Two-dimensional hierarchical clustering of the 1,222 *S. aureus* strains. The green/red figure represents 164,970 binary outcomes generated by high-throughput AFLP with 135 marker fragments per strain. Marker absence corresponds with green, and marker presence corresponds with red (gray represents ambiguous positions [i.e., weak bands] that are scored as marker absence in the mathematical analyses). The dendrogram on the *y* axis represents the phylogenetic clustering of the 1,222 strains. The dendrogram on the *x* axis shows the clustering of the 135 AFLP markers, many of which segregate in specific groups. The colored, striped bars on the right represent the distribution of the U.S. strains (blue) and the reference strains Mu50 and N-315 (light green). The Dutch carriage strains ($n = 829$) are not pointed out separately. In conjunction with PCA, four major branches (I, II, III, and IV) were identified; these are represented by the black and white bar on the right of the figure. AFLP cluster IVa has been annotated separately (between AFLP cluster I and II), because these *S. aureus* strains were assigned (arbitrarily) to cluster IV, based on PCA (Fig. 2 to 4).

DISCUSSION

In the United States, the overall nasal *S. aureus* colonization rate among humans was 32.4% (2001 to 2002) (3). This rate is significantly higher than the prevalence for patients admitted to Dutch hospitals (24.4%; $P < 0.0001$) (13). Whether this higher rate is due to the differences between the groups of volunteers from whom the strains were isolated or to different sampling methods is unknown. However, despite these differences in colonization rate and origin of the strains, the population structures of American and Dutch isolates are surprisingly similar.

The same major AFLP clusters (I, II, III, and IV) are identified in both Dutch ($n = 829$) and American ($n = 391$) community-based nasal isolates of *S. aureus*. Genetically heterogeneous AFLP cluster I was the largest cluster found, comprising nearly half of all MSSA isolates and the majority of the MRSA isolates. However, this cluster could be subdivided in several subclusters corresponding with different MLST clonal complexes. Notably, genetically homogeneous AFLP clusters II and III, which represent MLST clonal complexes 30 and 45, respectively, account for 46.4% of all carriage MSSA isolates in the current study population (47.3% of the Dutch carriage isolates and 44.0% of the American MSSA carriage isolates), suggesting that these two clonal complexes have evolved to be extremely successful in colonizing humans (6). It has been shown before that these two clonal types (CC30 and CC45), as found in The Netherlands, have a seemingly different potential to cause invasive infection (11). It would be worthwhile to seek confirmation of this observation in the American setting. This confirmation would contribute to our understanding of staphylococcal virulence traits in relation to their clonal backgrounds. Understanding this relationship may lead to optimized prophylactic and treatment options for the increasing burden of staphylococcal infections.

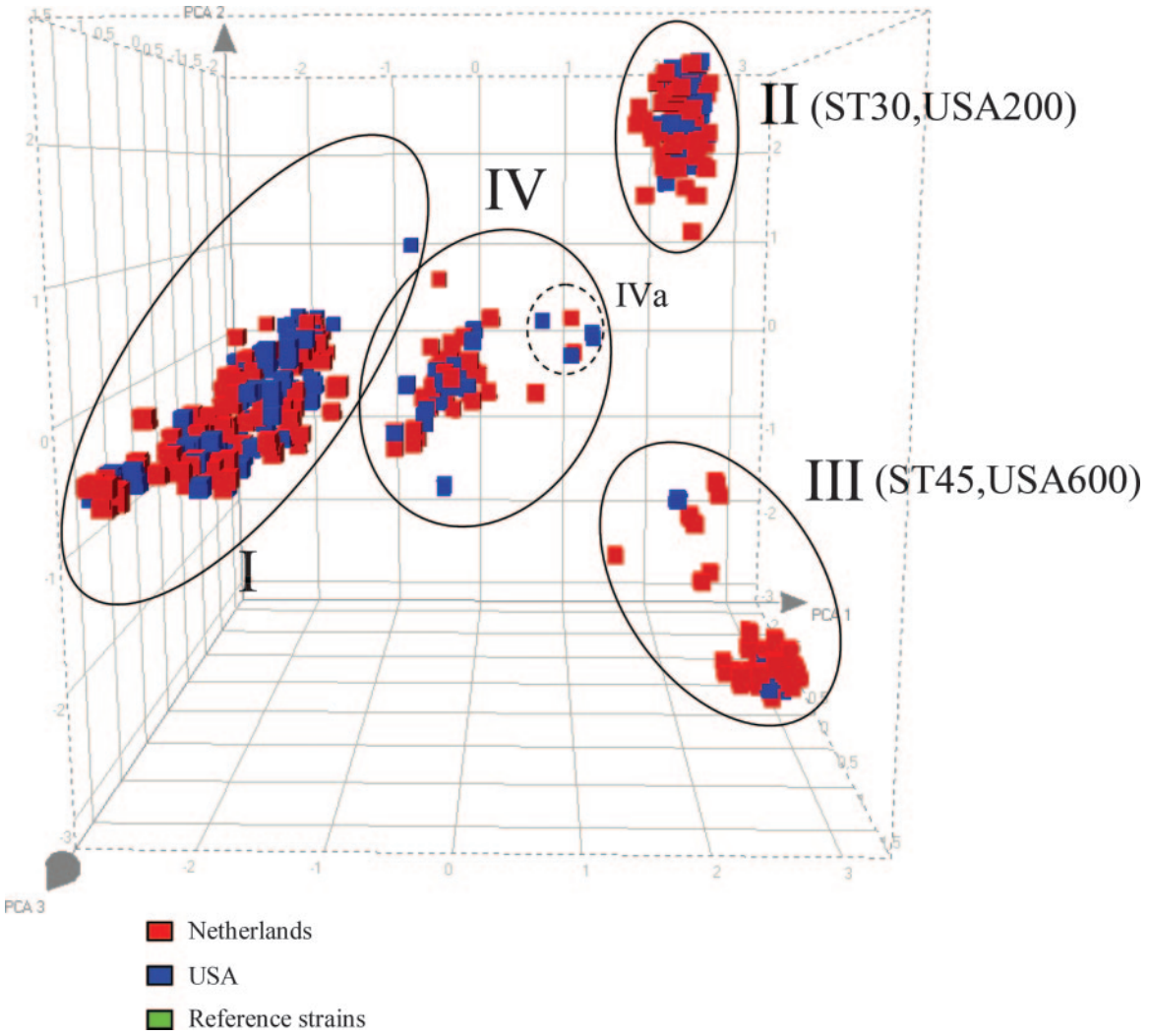

FIG. 2. PCA of the AFLP data for all 1,222 *S. aureus* isolates. The different cubes (plotted in three-dimensional space), colored according to the source, represent every strain in the study. Each axis represents the score calculated for that strain on each PC. The four circles indicate the different phylogenetic AFLP clusters. The two reference strains (N315 and Mu50) are not visible, as they are hidden by other strains.

TABLE 2. Distribution of *S. aureus* strains (n = 1220) in the four phylogenetic branches

AFLP cluster (clonal complex)	No. of strains (%) per AFLP cluster			
	Netherlands[a]	United States (MSSA)	United States (MRSA)	Total
I	367 (44.3)	140 (45.6)	76 (90.5)[b]	583 (47.8)
II (CC30)	216 (26.1)	95 (30.9)	2 (2.4)[c]	313 (25.7)
III (CC45)	176 (21.2)[d]	40 (13.0)[d]	1 (1.2)[e]	217 (17.8)
IV	70 (8.4)	32 (10.4)	5 (6.0)	107 (8.8)
Total	829 (100)	307 (100)	84 (100)	1,220 (100)

[a] All Dutch strains were MSSA.
[b] Proportionally, more of the MRSA strains in the U.S. cohort than of all the strains in the Dutch cohort (Fisher's exact test; $P < 0.0001$) or of the MSSA strains in the U.S. cohort (Fisher's exact test; $P < 0.0001$) were distributed into cluster I.
[c] Proportionally, fewer of the MRSA strains in the U.S. cohort than of all the strains in the Dutch cohort (Fisher's exact test; $P < 0.0001$) or of the MSSA strains in the U.S. cohort (Fisher's exact test; $P < 0.0001$) were distributed into cluster II.
[d] Proportionally, more of the carriage strains in the Dutch cohort than of the MSSA strains in the U.S. cohort (Fisher's exact test; $P = 0.0016$) were distributed into cluster III.
[e] Proportionally, fewer of the MRSA strains in the U.S. cohort than of all the strains in the Dutch cohort (Fisher's exact test; $P < 0.0001$) or of the MSSA strains in the U.S. cohort (Fisher's exact test; $P = 0.0005$) were distributed into cluster III.

Despite the strong similarity between both *S. aureus* population structures, there are also some differences. Dutch cluster III strains are overrepresented compared to their American counterparts ($P = 0.0016$). Furthermore, none of the Dutch nasal isolates included in the current study carried the *mecA* gene. In contrast, the MRSA prevalence in the American collection was approximately 2.5% ($P < 0.0001$) (3). However, it should be emphasized that methicillin resistance for the American isolates was tested for the total original collection, comprising approximately 3,000 *S. aureus* isolates. Methicillin resistance in the Dutch isolates was tested only for the 829 isolates included in the current study by *mecA* PCR, as described earlier (6). All MRSA strains isolated from the American cohort were included in the current study, in contrast to the American MSSA isolates, which were selected at random for the current study (by 1 out of 10). Therefore, we have compared the Dutch *S. aureus* isolates with the American MSSA and MRSA isolates separately. The American MRSA isolates were clearly overrepresented in major AFLP phy-

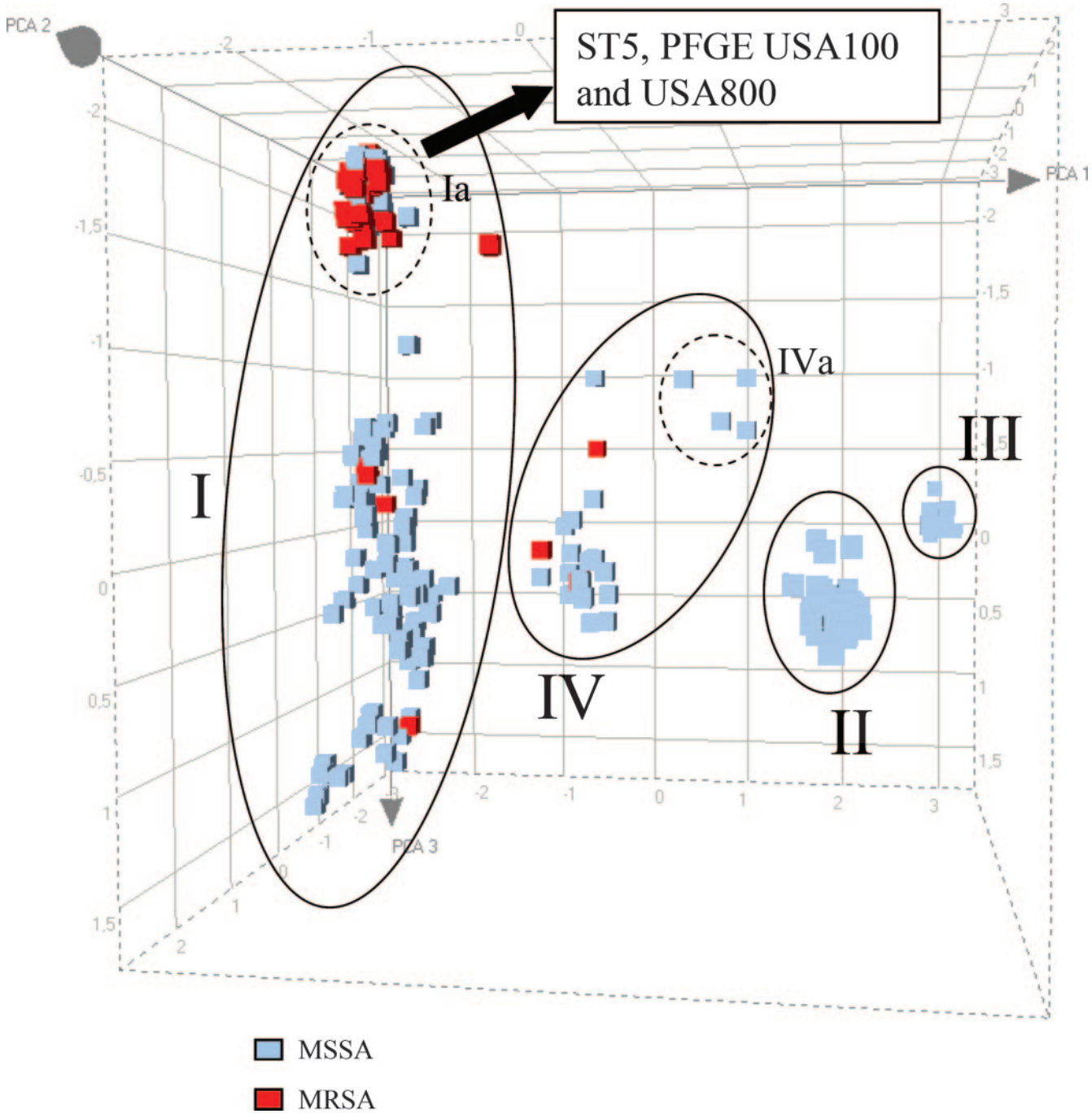

FIG. 3. Distribution of the American MSSA ($n = 307$) and the American MRSA ($n = 84$) isolates. PCA analysis of the AFLP data for all strains ($n = 1,222$), showing only the 391 American *S. aureus* isolates with the distribution of the MSSA versus the MRSA strains.

logenetic lineage I, and additionally, most of them clustered separately in a subcluster, named Ia. Our data clearly show that the American MRSA isolates (obtained from the open population) show significantly less genetic diversity than the MSSA carriage isolates included in this study, which is in agreement with the assumption that MRSA strains consist primarily of a limited number of highly successful pandemic clones.

PFGE typing showed that the American MSSA and MRSA isolates in AFLP subcluster Ia are PFGE type USA100 and USA800 strains. It is known that these clonal PFGE types share the same MLST sequence type (ST 5) and *spa* motif (MDMGMK). Apparently, these PFGE types share the same genetic background, which is corroborated by MLST and AFLP analysis. However, McDougal et al. earlier concluded that the two clonal PFGE types carry different SCC*mec* structures and had different susceptibility profiles (5). USA100 isolates cluster with representatives of the multire-

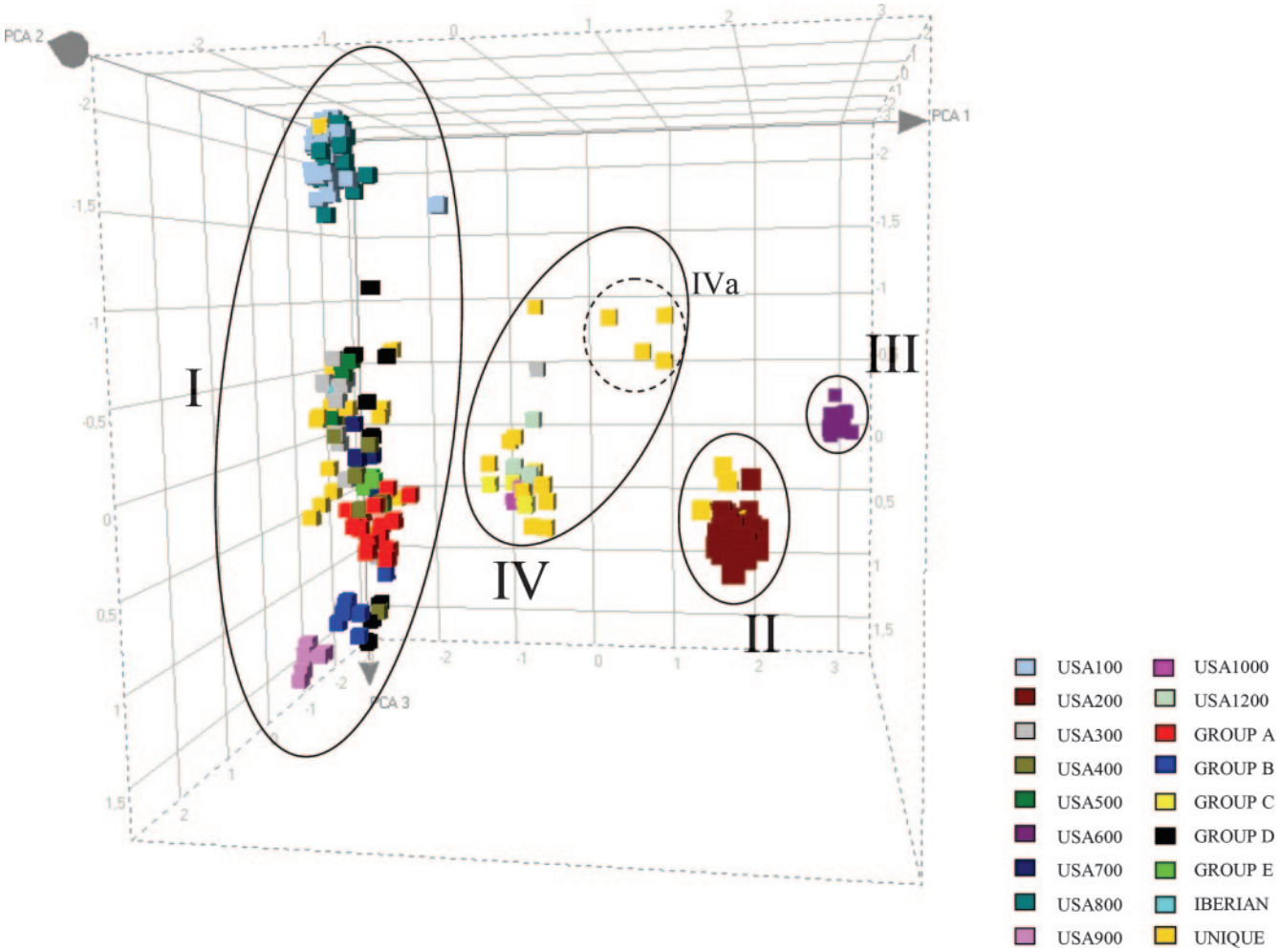

FIG. 4. Distribution of PFGE types of the 391 American *S. aureus* isolates over the four AFLP clusters. PCA of the AFLP data of all strains (n = 1,222), showing only the 391 American *S. aureus* isolates (which includes the American isolates that did not fit within the established USA PFGE lineages ["unique"]). The cubes are colored according to the PFGE type.

sistant New York/Japan clone containing SSC*mec* II, while isolates from USA800 cluster with representatives of the pediatric clone containing SSC*mec* IV (5). Thus, while these two lineages have diverged over time, they both appear to have retained the ability to colonize the nares of humans.

Finally, it should be emphasized that there are several differences in the demographics of the two populations surveyed, which could influence the results of this study. The Dutch *S. aureus* isolates were obtained from two separate cohorts, i.e., children under 19 years of age and elderly adults over 55 years of age, and all volunteers were living in the Rotterdam area (local region in The Netherlands). In contrast, the American *S. aureus* isolates were obtained from individuals covering all age groups and living all over the country.

In conclusion, the population structures of nasal *S. aureus* isolates from humans are congruent on both sides of the Atlantic. The same successful clones are present, although their relative frequencies may vary with geographic origin. Apparently, both MRSA and MSSA show similar epidemic behavior, with some clones being notably more successful colonizers of the human vestibulum nasi.

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