

Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*

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Article

Infectious disease

The population structure of *Staphylococcus aureus* carried by healthy humans was determined using a large strain collection of nonclinical origin ($n = 829$). High-throughput amplified fragment length polymorphism (AFLP) analysis revealed 3 major and 2 minor genetic clusters of *S. aureus*, which were corroborated by multilocus sequence typing. Major AFLP cluster I comprised 44.4% of the carriage isolates and showed additional heterogeneity whereas major AFLP groups II and III presented 2 homogeneous clusters, including 47.3% of all carriage isolates. Coanalysis of invasive *S. aureus* strains and epidemic methicillin-resistant *S. aureus* (MRSA) revealed that all major clusters contained invasive and multiresistant isolates. However, clusters and subclusters with overrepresentation of invasive isolates were also identified. Bacteremia in elderly adults, for instance, was caused by a IVa cluster-derived strain significantly more often than by strains from other AFLP clusters. Furthermore, expansion of multiresistant clones or clones associated with skin disease (impetigo) was detected, which suggests that epidemic potential is present in pathogenic strains of *S. aureus*. In addition, the virulence gene encoding Panton-Valentine leukocidin was significantly enriched in *S. aureus* strains causing abscesses and arthritis in comparison with the carriage group. We provide evidence that essentially any *S. aureus* genotype carried by humans can transform into a life-threatening human pathogen but that certain clones are more virulent than others.

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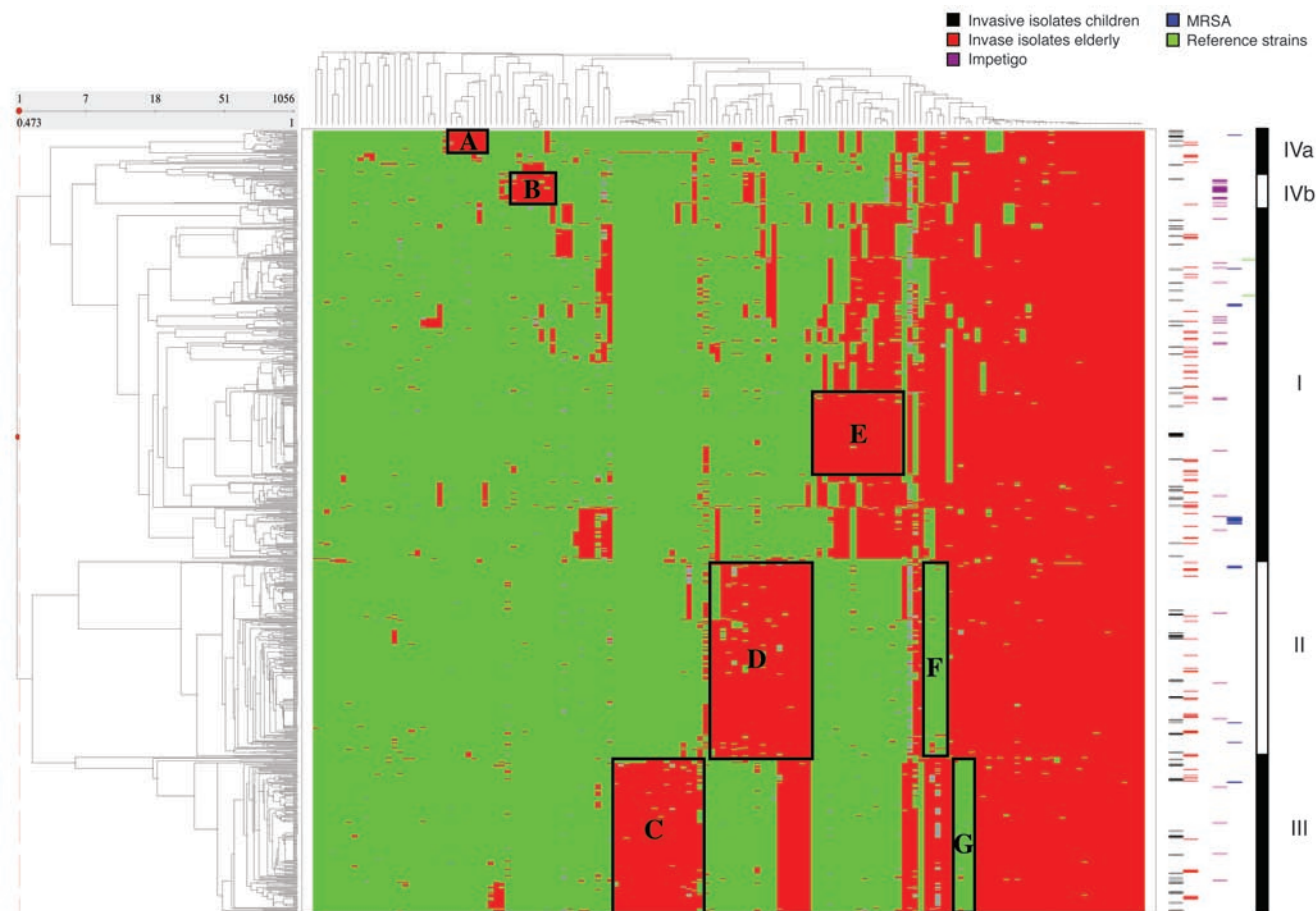


Figure 1

2D hierarchical clustering of the 1,056 *S. aureus* strains. The green/red figure represents 155,232 binary outcomes generated by ht-AFLP with 147 marker fragments. Marker absence corresponds with green and marker presence with red. The dendrogram on the y axis represents the phylogenetic clustering of the 1,056 strains. The dendrogram on the x axis shows the clustering of the 147 AFLP markers, many of which segregate in specific groups. These groups are cluster specific, and some of these groups are shown as boxes in the figure (A through G). The colored, striped bars on the right represent the distribution of the invasive strains (children and elderly adults), the MRSA strains, and the reference strains. The carriage strains ($n = 829$) are not pointed out separately. In conjunction with PCA 3 major (I, II, III) and 2 minor (IVa, IVb) branches were identified; these are represented by the black and white bar on the right of the figure.

We collected over 2,000 nonclinical *S. aureus* isolates from various groups of healthy individuals in the Dutch community over an extended period of time, creating a unique population-based strain collection. To assess differences in the virulence potential of various strains of *S. aureus*, insight into the natural (nonclinical) population structure is essential. We here present whole genome scanning by high-throughput AFLP (ht-AFLP) of a random selection of these *S. aureus* strains ($n = 829$). Strains were derived from the anterior nares of healthy children (1–18 years) and healthy elderly adults (>55 years) from the Rotterdam area (The Netherlands) (18, 19). To investigate genomic differences between these carriage strains and invasive isolates, contemporary isolates from blood, deep-seated, and soft-tissue infections from the same geographical area were included ($n = 164$). Furthermore, a collection of international epidemic MRSA strains ($n = 21$) (20) and *S. aureus* isolates from Rotterdam children with nonbulbous impetigo ($n = 40$) were included (21). The prevalence of the clinically relevant *mecA* and Panton-Valentine leukocidin (PVL) genes was determined for all strains as well.

Results

Genetic diversity of *S. aureus*. Using the set of 1,056 *S. aureus* strains, a total of 155,232 AFLP fragments were generated, covering 147 different marker fragments per strain. These outcomes are visualized in Figure 1. The dendrogram on the left (y axis) reveals bacterial clustering. The bar on the right of this figure delineates the presence of 3 major (I, II, III) and 2 minor (IVa, IVb) branches, as identified by principal component analysis (PCA) (Figure 2, A and B). Unsupervised cluster analysis of the 1,056 strains (Figure 3) clearly demonstrates that the AFLP data represent 2 distinct, homogenous major clusters (II and III) and, conversely, several other smaller subclusters that could be divided into 1 major and 2 minor AFLP clusters by PCA (I, IVa, IVb). MLST analysis indeed revealed additional heterogeneity in AFLP group I, identifying different clonal complexes (CCs) [CC5, CC8, CC15 (Figure 4)]. CCs are defined as clusters of closely related STs where single differences in the allelic profile are tolerated (9). In contrast, AFLP clusters II and III harbor single CCs, CC30 and CC45, respectively. These 2 major CCs embrace almost half (47.3%) of all carriage isolates. Clusters IVa and

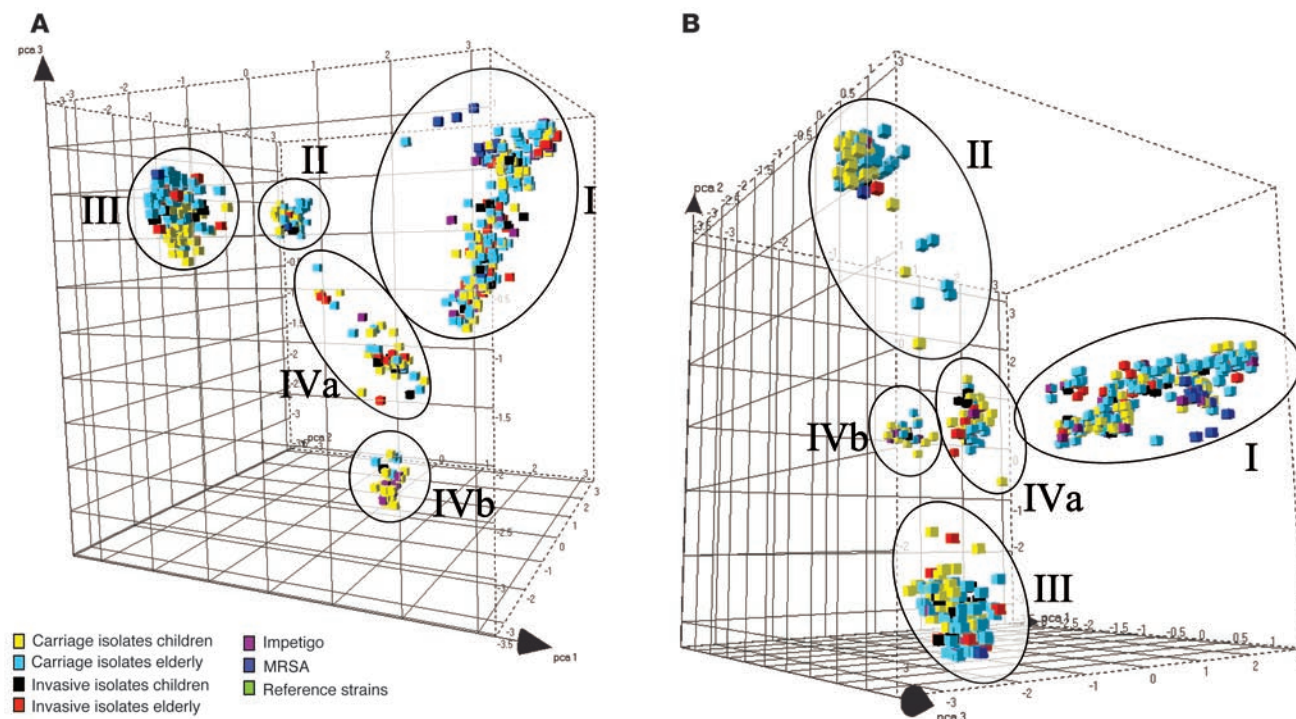


Figure 2

PCA of the 1,056 *S. aureus* strains. The different cubes (plotted in 3D 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 distribution of the strains is shown from 2 different angles (A and B). The 5 circles indicate the different phylogenetic AFLP clusters.

IVb are associated with CC22 and CC121, respectively (Figure 4). The unsupervised correlation-analysis showed that minor AFLP group IVa also consists of different subclusters (Figure 3).

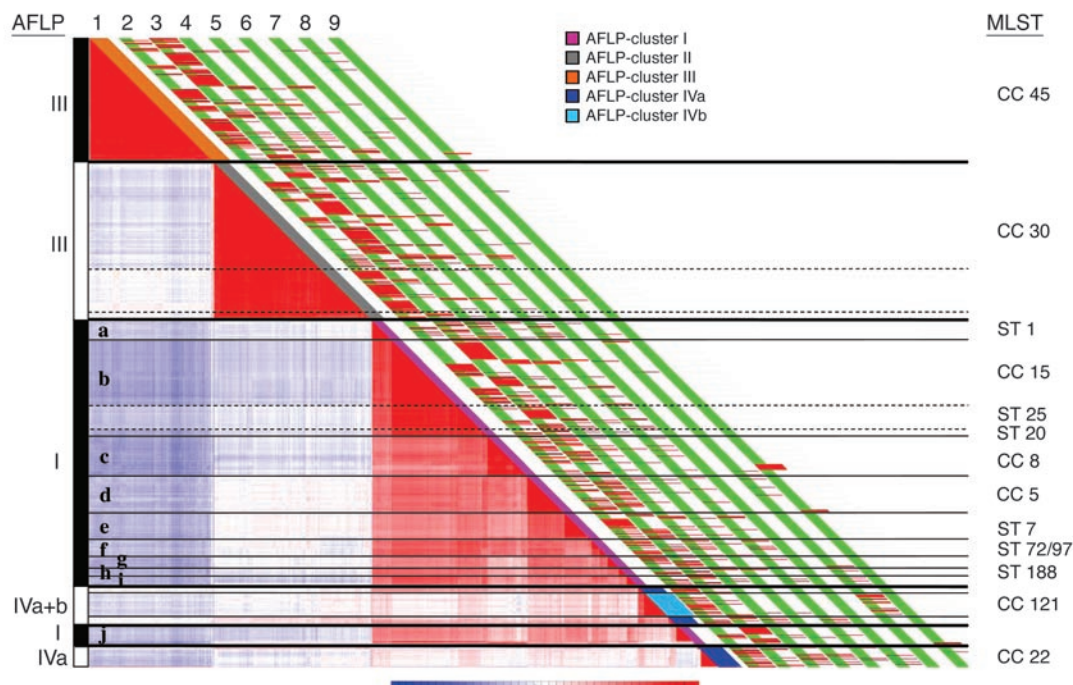
The horizontal dendrogram in Figure 1 shows the clustering of the 147 AFLP markers, many of which segregate in separate groups. These groups are cluster specific; some of these are shown as boxes A–G (Figure 1). The colored bars on the right represent the distribution of the invasive strains (in children and elderly adults), the impetigo isolates, the MRSA strains, and the reference strains across the dendrogram. The central area of this figure best defines the 5 phylogenetic lineages. The clustered green and red boxes show components of genomic diversity within the 5 *S. aureus* subpopulations. The distributions of the carriage isolates as well as the invasive isolates are also visualized in Figure 3.

Carriage versus disease-causing strains of *S. aureus*. Among the *S. aureus* isolates from healthy individuals with nasal carriage, 3 major genetic clusters (I, II, III) could be identified, comprising 760 of the 829 (91.7%) carriage strains. Two minor clusters (IVa, IVb) embrace the remaining 69 (8.3%) carriage strains. All 5 clusters coherently contain carriage strains isolated from children as well as elderly adults (Figure 5). The distribution of the pediatric and geriatric strains across the 5 AFLP clusters was overlapping (Figure 3). However, healthy children more often carried strains from cluster IVa+b than did healthy elderly adults (Fisher's exact test, $P < 0.0001$), whereas cluster I strains were slightly overrepresented in elderly adults (Fisher's exact test, $P = 0.03$). Although carriage isolates from children and elderly adults were equally divided in AFLP cluster II (Figure 5), a clone strongly associated with carriage isolates in children could be identified (Fisher's exact test, $P < 0.0001$) (area between the dotted lines in Figure 3).

Contemporary invasive *S. aureus* strains ($n = 164$) from children and elderly adults were distributed across all phylogenetic branches and widely scattered across the AFLP dendrogram (Figures 1 and 3). The population structure of contemporary carriage isolates and invasive isolates in the same geographical area appeared to be strongly overlapping (Figure 5). However, bacteremia in elderly adults was significantly more often caused by a IVa cluster-derived strain (Fisher's exact test, $P = 0.0095$). Additional analysis of AFLP cluster IVa showed that this group consists of distinct subclusters (Figure 3). Therefore it would be incorrect to define this minor cluster as a single invasive clone. Apparently, several subclusters, plotted next to each other by 3D (AFLP cluster IVa), contain proportionately more bacteremia isolates from elderly adults than carriage isolates from the same group. Statistical analysis of major cluster I revealed 2 subclusters potentially associated with invasive disease. Subcluster If (Figure 3) contains proportionately more bacteremia isolates from elderly adults ($n = 5$; 5.6%) in comparison with carriage isolates from elderly adults ($n = 6$; 1.4%) (Fisher's exact test, $P = 0.027$). Subcluster Ia shows overrepresentation of invasive isolates from both children and elderly adults ($n = 11$; 6.7%) in comparison with carriage isolates from both groups ($n = 20$; 2.4%) (Fisher's exact test; $P = 0.01$) (Figure 3).

The invasive strains from children in major cluster I are associated with hospital-acquired disease (Fisher's exact test, $P = 0.01$) (data not shown). No significant difference was found in the distribution of isolates from individuals with invasive community-acquired disease versus invasive hospital-acquired disease in the other clusters.

All 5 clusters contain *S. aureus* strains isolated from children with community-acquired nonbullous impetigo. The distribution is shown in Figures 1 and 5. Compared to pediatric carriage strains,

**Figure 3**

Cluster analysis of the 1,056 *S. aureus* strains using OmniViz. The cells in the correlation visualization are colored by Pearson's correlation coefficient values with deeper colors indicating higher positive (red) or negative (blue) correlations. The scale bar (underneath the figure) indicates 100% correlation (red) toward 100% anticorrelation (blue). In order to reveal correlation patterns, a matrix-ordering method was applied to rearrange the samples. The OmniViz correlation view generated with 1,056 strains was adapted so that descriptive (clinical) parameters could be plotted directly adjacent to the original diagonal. The black and white bar on the left indicates the 5 AFLP groups based on PCA. This figure shows additional subclustering in major group I (a–j) as well as in minor group IVa, indicated by several lines. The dotted lines identify blocks of minimal changes in 1 cluster or subcluster. The corresponding MLST data (see also Figure 4) are shown on the right side of the figure. The distributions of the strains from different origins are visualized as red lines in the diagonal red and green bars of the figure (numbered 2–9). Variable 1 indicates the different AFLP clusters based on PCA; 2, carriage isolates, children ($n = 400$); 3, carriage isolates, elderly adults ($n = 429$); 4, invasive isolates, total ($n = 164$); 5, invasive isolates, children ($n = 74$); 6, invasive isolates, elderly adults ($n = 90$); 7, invasive isolates, children (deep-seated and soft-tissue infections) ($n = 18$); 8, impetigo isolates ($n = 40$); 9, MRSA ($n = 21$).

impetigo isolates were more frequently found in cluster IVb (Fisher's exact test, $P < 0.0001$) and less frequently in cluster II (Fisher's exact test, $P = 0.01$) (Figure 5), suggesting clonal expansion of a certain genotype associated with impetigo (22).

***MecA*- and *PVL*-positive strains.** The 21 international epidemic MRSA strains are present in several clusters and subclusters of *S. aureus* (Ic, Id, Ij, II, III, IVa). This strain collection comprises epidemic MRSA from Belgium, Finland, France, Greece, Spain, Germany, and the United Kingdom (20). AFLP cluster I contained disproportionately more MRSA isolates as compared to the other clusters (Fisher's exact test, $P = 0.006$) (Figure 5), suggesting that many of these epidemic MRSA strains are derived from a common cluster I ancestor. Notably, most of the 21 MRSA strains are located in subcluster Ic ($n = 9$; 42.9%; Fisher's exact test, $P < 0.0001$).

None of the Dutch carriage and clinical *S. aureus* isolates ($n = 1033$) included in this study harbor the *mecA* gene, which is consistent with the reported low MRSA prevalence in the Dutch population (23). Four *PVL*-positive *S. aureus* strains (1.0%) were found in the pediatric carriage group ($n = 400$) and 1 (0.2%) in the elderly adults carriage group ($n = 429$). Three of 146 (2.1%) blood-culture isolates carried the *PVL* gene, 2 of which derived from pediatric patients. Seven of the 18 (38.9%) invasive strains isolated from deep-seated or soft-tissue infections in children were *PVL* positive. There was no significant difference in the presence of *PVL* when comparing the carriage

isolates and invasive blood-culture isolates. In contrast, *S. aureus* strains causing abscesses and arthritis were significantly enriched in the presence of *PVL* (38.9%) in comparison with the pediatric carriage group (1.0%) (Fisher's exact test, $P < 0.0001$) and in comparison with the pediatric bacteremia isolates (3.6%) (Fisher's exact test, $P = 0.0005$). All impetigo strains ($n = 40$) were *PVL* negative.

Sequence assessment of AFLP markers. To determine the origin of genetic polymorphism, the nucleotide sequence of a set of 81 AFLP markers was established, 60 of which were located in cluster-specific marker boxes (Figure 1, boxes A–G). Nineteen of the remaining 21 markers were not cluster specific and were present in almost all 1,056 *S. aureus* strains (red area on the right side of box G, Figure 1). Interestingly, 66 (81.5%) of the 81 markers showed homology to all 3 completed (MW2, Mu50, N315) and 4 unfinished (252, 476, COL, NCTC 8325) genome sequences of *S. aureus*. Conversely, 7 (8.6%) of the 81 markers appear to be absent in all 3 completed genomes (MW2, Mu50, N315) of *S. aureus* (see Supplemental Table 1; supplemental material available at <http://www.jci.org/cgi/content/full/114/12/1732/DC1>). Of these 7 marker fragments, 3 showed homology to the *S. aureus* strain 252 epidemic MRSA-16 (EMRSA-16), 1 to bacteriophage Φ exfoliative toxin A (ETA) DNA, and 1 to *S. aureus* TY4 exfoliative toxin B (ETB) plasmid DNA. Only 2 did not match with any GenBank entry and may represent novel (or hypermutable) *S. aureus* genome segments. The 15 markers that

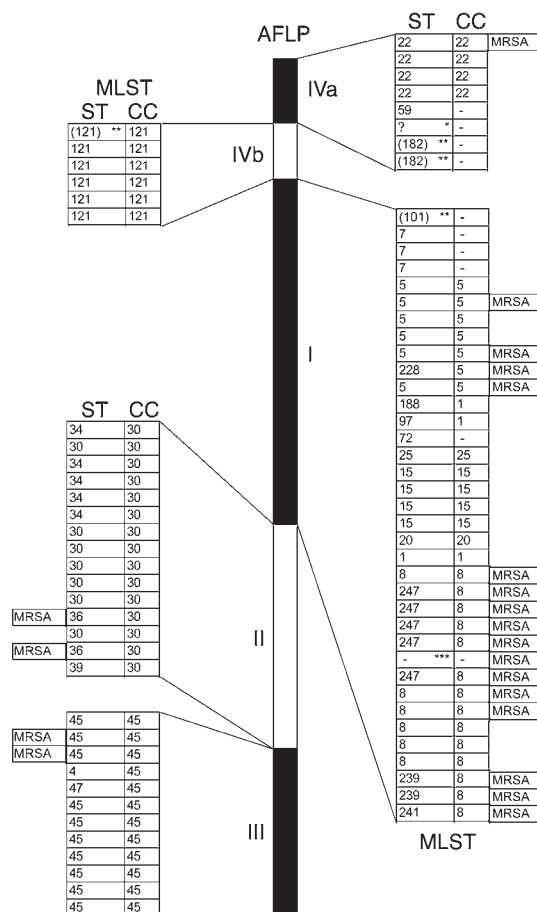


Figure 4

AFLP analysis versus MLST analysis of *S. aureus*. The bar in the center of the figure represents the 1,056 strains divided into the 5 phylogenetic AFLP clusters (similar to those defined at the right side of Figure 1). MLST data is shown for 77 *S. aureus* strains, which are spread over the different AFLP clusters. The order of the MLST sequence types in this figure is determined by the location of the strain in the AFLP dendrogram (Figure 1). *Unknown ST; **6 of the 7 loci are similar to the particular ST; ***data not available.

may be explained by host susceptibility and population aging, this does not fully explain the current and drastic rise in the number of infections. However, little is known about possible bacterial determinants and whether or not these are associated with changes in the virulence of *S. aureus*.

We previously showed that AFLP analysis using optimal enzyme and primer combinations is an excellent tool for assessing genetic polymorphism in the clonal microorganism *M. tuberculosis* (17). For *S. aureus* we used the enzyme combination *MboI*-*Csp6I*, which resulted in a fingerprint of about 70 polymorphic AFLP fragments well distributed within the size range of 100 bp to 600 bp in a single AFLP reaction. However, a potential limitation of this AFLP approach is in the randomness of the restriction sites for *MboI* and *Csp6I*. For instance, genomic islands with underrepresentation of these restriction sites will not be fully scanned for polymorphism. However, if currently known genomic sequences of *S. aureus* (Mu50 and N315) are analyzed by computer for the occurrence of these sites, the average number of fragments (useful for AFLP) generated per genome is 4.373, and the average length of the fragments is 200 bp. This suggests that coverage is indeed random and, in this respect, AFLP provides more of a whole genome-scanning approach than MLST, for instance.

In the present study, the population structure of *S. aureus*, isolated from the nose of healthy individuals in the Rotterdam area (The Netherlands), has been determined. Using ht-AFLP, we analyzed 147 polymorphic markers for 1,056 *S. aureus* strains. Two large unbiased strain collections of a nonclinical origin were used. These collections were obtained from children (<19 years) and elderly adults (>55 years) with nasal carriage of *S. aureus*. ht-AFLP analysis revealed the existence of 3 major (I, II, III) and 2 minor (IVa, IVb) phylogenetic branches. Major AFLP group I could be subdivided into 10 different subclusters, indicating its heterogeneity. In contrast, major clusters II and III showed a very homogenous nature.

Carriage isolates were not completely randomly distributed over these clusters. AFLP cluster IVa+b represents more carriage strains from children (isolated in 2002) than carriage strains from elderly adults (isolated from 1997–1999). A clonal expansion associated with carriage isolates in children was also observed in AFLP group II. Conversely, AFLP group I embraces more carriage strains from elderly adults. Apparently, a certain degree of bacterial population heterogeneity exists between the 2 groups included. Whether the different sampling moments or the different age categories are fundamental to the observed differences is currently unclear. Danish studies from the late 1970s demonstrated that waves of phage types of *S. aureus* go through human populations (25, 26). Such a phenomenon may explain our current observations. However, the proportionate distribution of the major phylogenetic branches (I, II, III) within the overall population of *S. aureus*, isolated from humans, appears to be fairly stable over time and comparable for children and elderly adults.

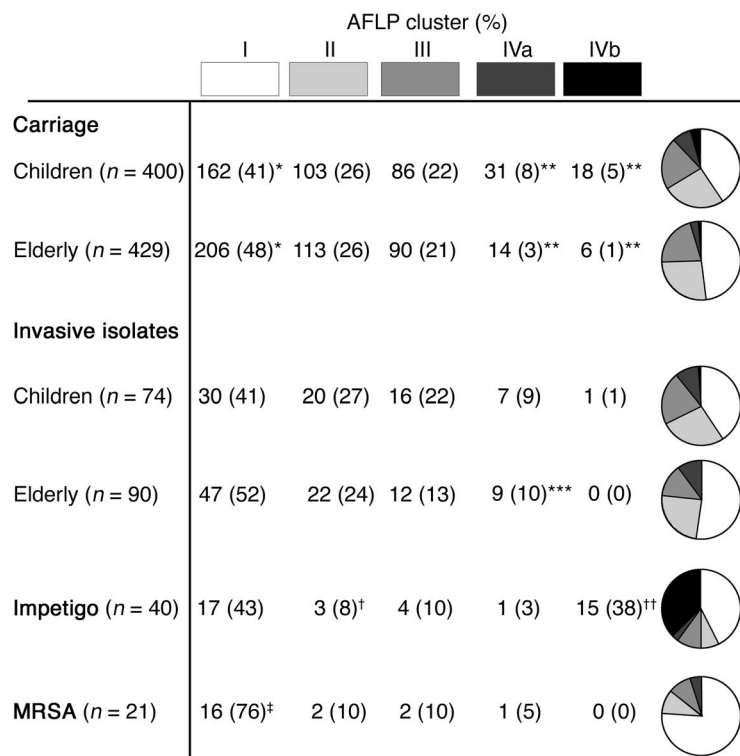
appear to be absent in at least 1 of the known genome sequences of *S. aureus* may be associated with (larger) deletions, the existence of which has been described before (24).

The cluster-specific marker boxes are associated with different AFLP clusters. Box A is associated with a part of cluster IVa, B with cluster IVb, C (present) and G (absent) with cluster III, D (present) and F (absent) with cluster II, and box E with cluster I (Figure 1). The majority (78.0%) of the markers present in boxes A–D (not present in cluster I) showed homology to the reference strains Mu50 and N315, which are located in AFLP cluster I. This suggests that these markers become cluster specific by point mutations rather than genomic rearrangement (deletions or insertions). This is also supported by additional data (Supplemental Figure 1), which show that cluster-defining markers in each box are widely spread across the *S. aureus* chromosome.

Cluster-specific box B, associated with cluster IVb (with overrepresentation of impetigo-isolates), comprises both virulence genes bacteriophage Φ ETA DNA and ETB plasmid DNA (Supplemental Table 1).

Discussion

The incidence of *S. aureus* bacteremia is rising and has more than doubled over the past 25 years in some Western European countries. This increase coincides with a growing rate of community-acquired disease (in proportion to hospital-acquired disease) and the epidemic emergence of nosocomial MRSA strains (2). Elderly adults are most frequently affected, particularly those with additional predisposing risk factors. Although a part of this effect

**Figure 5**

Distribution of *S. aureus* strains in the 5 phylogenetic branches. *Overrepresentation of carriage in elderly adults (Fisher's exact test, $P = 0.01$); **overrepresentation of carriage in children (Fisher's exact test, $P < 0.0001$); ***proportionately more bacteremia-associated strains from elderly adults as compared to carriage strains from the same group (3% vs. 10%; Fisher's exact test, $P = 0.0095$); [†]proportionately fewer impetigo-associated strains as compared to carriage in children (8% vs. 26%; Fisher's exact test, $P = 0.01$); ^{††}overrepresentation of impetigo-associated strains as compared to carriage in children (38% vs. 5%; Fisher's exact test, $P < 0.0001$); [‡]proportionately more MRSA strains as compared to all carriage isolates (76% vs. 44%; Fisher's exact test, $P < 0.006$).

The 5 AFLP clusters identified in this study match with the major CCs as defined by MLST (<http://www.mlst.net/>). These MLST-based CCs have been defined by studying carriage, invasive, and MRSA isolates mainly from the United Kingdom. The top 5 CCs in the MLST database are CC8, CC30, CC5, CC22, and CC45 (9, 15, 27, 28). We studied more than 1,000 strains isolated in the Rotterdam region (The Netherlands), and we identified essentially the same CCs. Apparently, these clonal clusters have spread successfully in the United Kingdom and The Netherlands and probably worldwide. All large-scale molecular typing studies of nonclinical isolates of *S. aureus* have been performed using geographically biased strain collections, including our present analysis. However, considering the overlap in MLST types and the similarity in prevalence of certain major clonal clusters, it is supposed that geographical bias is not a confounding factor. An ongoing analysis of strains derived from Indonesian carriers corroborated this hypothesis. The Indonesian strains clustered in the same groups (I to IV), although there was a difference in the relative numbers of isolates per cluster. No new AFLP clusters were identified (Melles et al., unpublished data).

ht-AFLP clusters II and III, identical to MLST CCs 30 and 45, respectively, account for almost half (47%) of all carriage isolates in our population, which suggests that these 2 CCs have evolved to be very successful in colonizing humans.

The above-mentioned observations suggest that virulent and nonvirulent strains are probably not fundamentally different from each other: clinical isolates and MRSA from international sources fall into the same main clusters as carriage isolates. In addition, invasive *S. aureus* strains, mainly blood-culture isolates (89%), were found to be widely distributed across all 5 AFLP groups in this study. This suggests that strains from each of the genetic clusters are essentially able to cause invasive disease. On the other hand, 1 minor AFLP cluster (IVa) and 2 AFLP subclusters (Ia and If) contained proportionately more invasive isolates. Direct

cross-infection is not a likely explanation for this finding because these epidemiologically nonrelated invasive strains were isolated from patients in different medical departments over a period of several years. There have been controversial reports on the existence of hypervirulent lineages of *S. aureus* (9, 12), but our data suggest that not all *S. aureus* strains share the same invasive potential. This is not in agreement with recent findings from the Oxford (United Kingdom) region where MLST data suggest that virulence may not be associated with clonal lineages (15). Rather, a relationship between genetic background and disease type is thought to be primarily dependent on the presence of certain toxin genes only (29).

Table 1Number of *Staphylococcus aureus* strains included in this study

	Children (<i>n</i>)	Elderly adults (<i>n</i>)	Total (<i>n</i>)
Carriage	400	429	829
Invasive — blood culture			
Hospital acquired	43	68	111
Community acquired	13	22	35
Invasive — deep-seated or soft-tissue infection			
Hospital acquired	4	—	4
Community acquired	12	—	12
Unknown	2	—	2
Impetigo	40	—	40
MRSA	—	—	21
Reference strains	—	—	2
Total	514	519	1056



S. aureus strains isolated in the Rotterdam area from patients with nonbullous impetigo showed less clonal diversity than bacteremia-associated strains. Although each of the 5 clusters was found to contain impetigo-derived isolates, AFLP cluster IVb clearly represented significantly more isolates causing impetigo. An explanation for this clonal expansion in impetigo could be the facile spread of this disease. A recent study by Koning et al. concerning nonbullous *S. aureus* impetigo concluded that a combination of staphylococcal virulence and resistance genes determines the development and course of nonbullous impetigo (22).

The 21 international epidemic MRSA strains included in this study were spread across several lineages, indicating that methicillin resistance has developed in distinct phylogenetic subpopulations of *S. aureus*, which has been described before (10, 30). MLST studies have placed most of the MRSA in 5 major clusters: CC5, CC8, CC22, CC30, and CC45 (10, 27, 31, 32). Figure 4 shows similar dissemination for strains from a nonclinical origin as well. Notably, computer algorithms used to solve the *S. aureus* population structure based on MLST data (eBURST; ref. 28) generate clustering identical to that found in the AFLP Spotfire and OmniViz analyses. This provides a solid experimental and mathematical framework for all conclusions drawn from our AFLP data.

We have also assessed the prevalence of *mecA* and the PVL genes in the carriage and disease-causing populations of *S. aureus*. All carriage isolates ($n = 829$) were *mecA* negative, corroborating data showing the insignificant spread of MRSA in the Dutch community (23). Also, all clinical isolates ($n = 204$) were *mecA* negative, as opposed to proportions of above 10% in many of the other European countries, including those sharing borders with The Netherlands (33). PVL is a toxin associated with skin infections (furuncles), community-acquired MRSA infections, and necrotizing pneumonia (31, 34, 35). PVL prevalence in a *S. aureus* population of nonclinical isolates has never been studied accurately. We found a very low prevalence of 0.6% in a large ($n = 829$) carriage collection. In this study PVL was carried in 2.1% of blood-culture isolates. However, a significantly higher prevalence of PVL (38.9%) was found in *S. aureus* strains causing abscesses and arthritis. This is in agreement with the proposed involvement of PVL in severe and invasive (soft-tissue) staphylococcal infections (31, 35).

Our sequence analysis for the clustered AFLP markers suggested that genetic diversity among clusters is primarily caused by point mutation rather than by large-scale deletions or insertions (15). Ultimate proof for this hypothesis should be provided by detailed physical mapping and large-scale sequencing studies, however. Furthermore, we provide indirect proof that 7 genome sequences quite accurately represent the genetic potential of *S. aureus* as a species; only 4 of 81 marker sequences did not match with the 7 known *S. aureus* whole genome sequences.

In conclusion, we have solved the population structure of *S. aureus* of nonclinical origin. Three major and 2 minor phylogenetic branches were identified in our geographically restricted group. Inclusion of invasive *S. aureus* strains and international-epidemic MRSA revealed that within all major clusters, invasive and multiresistant isolates could be identified. However, clusters and subclusters with overrepresentation of bacteremia-associated isolates were identified. Expansion of multiresistant clones or clones associated with skin disease (impetigo) was observed as well. We suggest that essentially any *S. aureus* genotype that is carried by humans can transform into a life-threatening human pathogen, but strains from some clonal lineages are more virulent than others.

Methods

Bacterial strains. Two strain collections provided nonclinical *S. aureus* carriage isolates from healthy individuals. These collections were obtained from 2 study cohorts involving children and elderly adults. In addition, various clinical isolates were included. Contemporary invasive *S. aureus* strains, isolated from children and elderly adults from the same geographic region, were cultured from normally sterile sites in hospitalized patients with clinical signs of *S. aureus* infection. Community-acquired invasive disease was defined as isolation of *S. aureus* from patients within 48 hours of admission; hospital-acquired was defined as isolation of *S. aureus* 48 hours or longer after admission. The different subcollections are described in more detail below.

In total, 3,198 children from Rotterdam (The Netherlands), aged between 1 and 19 years and participating in the national 2002 Meningococcal Vaccination Campaign, were enrolled (19). A team of 10 research nurses and medical doctors obtained a single nasopharyngeal swab per child at the time of vaccination. *S. aureus* was isolated from 1,116 children. All isolates were stored at -80°C in broth containing glycerol. A random sample of 400 *S. aureus* carriage isolates was drawn.

The second collection originated from a community-based prospective study of elderly adults in Rotterdam (The Netherlands) (18). From 3,851 persons aged over 55 years, nasal swab cultures were obtained between April 1, 1997, and December 31, 1999. *S. aureus* strains were isolated from 1,043 elderly adults. All isolates were stored at -80°C in glycerol containing broth. A random sample of 429 carriage isolates was drawn.

Seventy-four clinical *S. aureus* isolates were retrospectively collected from children with invasive *S. aureus* disease identified in Sophia Children's University Hospital (Rotterdam, The Netherlands) (2000–2002). Fifty-six isolates derived from blood cultures and 18 isolates were obtained from deep-seated (arthritis; $n = 4$) or soft-tissue (abscess; $n = 14$) infections. Ninety clinical isolates from elderly adults (>55 years) were obtained from persons with *S. aureus* bacteremia identified in Erasmus MC (Rotterdam, The Netherlands) (1997–1999). Forty *S. aureus* strains obtained from lesions of children suffering from impetigo were randomly drawn from a collection described by Koning et al. (21).

Twenty-one international epidemic MRSA strains were obtained from the HARMONY collection (<http://www.harmony-microbe.net>), described by Murchan et al. (20). Finally, we included 2 reference strains. N315 is an MRSA strain isolated in 1982, and Mu50 is an MRSA strain with reduced susceptibility to vancomycin isolated in 1997. For both strains genome sequences have been determined (36). In total, 1,056 *S. aureus* strains were included (Table 1).

Cultures, DNA isolation, and detection of *mecA* and PVL genes. Bacteria were grown overnight at 37°C on Columbia III agar (BD) supplemented with 5% sheep blood. Three to 5 colonies were suspended in TEG buffer (25 mM Tris, 10 mM EDTA, 50 mM glucose) containing lysostaphin (50 $\mu\text{g}/\text{ml}$) and incubated at 37°C for 1 hour. DNA was extracted with the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) using the MagNA Pure LC Instrument (Roche Diagnostics) and stored at -20°C . We determined the presence of the *mecA* and PVL genes by PCR (34, 37).

AFLP. AFLP analysis has been performed as described by Van den Braak et al. (17). Using the predictive software package Recomb (Keygene NV) (38), the optimal enzyme and primer combinations were selected. Bacterial DNA was digested with the enzymes *MboI* and *Csp6I*, and the linker oligonucleotide pair for *MboI* (5'-CTCGTAGACTGCGTACC-3' and 5'-GATCGGTACGAGTC-TAC-3') and for *Csp6I* (5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAG-GACTCAT-3') were ligated. Subsequently, a nonselective preamplification was performed using the *MboI* primer (5'-GTAGACTGCGTACCGATC-3') and *Csp6I* primer (5'-GACGATGAGTCCTGACTAC-3'). In the final amplification, a ^{33}P -labeled *MboI* primer containing 1 selective nucleotide (either +C or +G) and a *Csp6I* primer containing 2 selective nucleotides (+TA) were used. Amplified material was analyzed using standard polyacrylamide slab gels and



subsequent autoradiography. Marker fragments were scored and a binary table scoring marker fragment absence (0) or presence (1) was constructed.

After excision of some selected AFLP fragments from dried gels, reamplification followed by double-strand sequence analysis was performed (17, 38). The sequence of several additional fragments was determined by computer analysis. The size of the fragments in combination with the selective nucleotides of the AFLP primers facilitated adequate mapping of the fragments on the staphylococcal genome sequence. These fragments were further analyzed by BLAST searching (<http://www.ncbi.nlm.nih.gov/BLAST/>) (39) against the 3 completed (MW2, NC_003923.1; Mu50, NC_002758.1; N315, NC_002745.2) and 4 unfinished (252, NC_002952; 476, NC_002953; COL, NC_002951; NCTC 8325, NC_002954) genomic sequences of *S. aureus*. The BLAST results enabled computer-mediated genomic localization and gene annotations of the AFLP fragments.

MLST. MLST was carried out for 56 *S. aureus* strains using DNA arrays (40). The selected strains were equally distributed across the AFLP dendrogram by selecting 1 out of 10 carriage or invasive strains isolated from children, going from top to bottom through the AFLP dendrogram (Figure 1). MLST data for the 21 epidemic MRSA strains are available at the MLST home page (<http://www.mlst.net/>) (20).

Data analysis. The method used for 2D clustering of the AFLP data was agglomerative (successive) hierarchical. This was performed using the unweighted pair group method with arithmetic mean (UPGMA). The similarity metric used was Tanimoto (Spotfire DecisionSite 7.2; Spotfire), which defines similarity for binary data (0 and 1) based on the number of positive attributes that 2 records have in common. The resulting dendrogram was ordered by average value.

PCA is a standard multivariate method used to reduce the dimensional space of the data to its principal components (PCs) (41, 42). PCA aims to reduce a large number of variables that explain most of the variation in the data (43). It is basically a rotation of axes after centering data to the means of the variables, the rotated axes being the PCs, which are linear combinations of the original variables. The PC computation is displayed as a 3D 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 2D representation of hierarchical clustering. The distribution of the strains in the 5 phylogenetic branches was defined on the basis of PCA. Hierarchical cluster analysis and PCA were performed using Spotfire DecisionSite 7.2 software.

The OmniViz package (OmniViz Inc.) was used to perform and visualize the results of unsupervised cluster analysis in a correlation visualization. This correlation visualization tool displays pairwise correlations among the samples calculated by Pearson's correlation coefficient (44). In order to reveal correlation patterns, a matrix-ordering method is applied to rearrange the samples. The ordering algorithm starts with the most correlated sample pair and, through an iterative process, sorts all the samples into correlated blocks. Each sample is joined to a block in an ordered manner so that a correlation trend is formed within a block with the most correlated samples at the center. The blocks are then positioned along the diagonal of the plot in a similar ordered manner. As the resultant visualization is symmetrical about the diagonal, half the matrix display is replaced by appropriate clinical data.

To compare the distribution of strain categories in different phylogenetic lineages, Fisher's exact test was used. A 2-sided *P* value of less than 0.05 was considered significant.

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