Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*

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A small number of clonal lineages dominates the global population structure of methicillin-resistant *Staphylococcus aureus* (MRSA), resulting in the concept that MRSA has emerged on a few occasions after penicillinase-stable β-lactam antibiotics were introduced to clinical practice, followed by intercontinental spread of individual clones. We investigated the evolutionary history of an MRSA clone (ST5) by mutation discovery at 108 loci (46 kb) within a global collection of 135 isolates. The SNPs that were ascertained define a radial phylogenetic structure within ST5 consisting of at least 5 chains of mutational steps that define geographically associated clades. These clades are not concordant with previously described groupings based on staphylococcal protein A gene (*spa*) typing. By mapping the number of independent imports of the staphylococcal cassette chromosome methicillin-resistance island, we also show that import has occurred on at least 23 occasions within this single sequence type and that the progeny of such recombinant strains usually are distributed locally rather than globally. These results provide strong evidence that geographical spread of MRSA over long distances and across cultural borders is a rare event compared with the frequency with which the staphylococcal cassette chromosome island has been imported.

antibiotic resistance | evolution | phylogeography | SNP

The Gram-positive bacterium *Staphylococcus aureus* is a leading cause of hospital-acquired infections that results in disease in 2% of all newly admitted patients (1). It is the most frequent cause of surgical site infections, lower respiratory tract infections, and cardiovascular infections and is the second most common cause of health care–associated pneumonia and bloodstream infections (2, 3). According to recent estimates, >400,000 *S. aureus*–related hospitalizations occurred per year in the United States, causing 11,000 deaths annually (4). The ability of *S. aureus* to develop resistance to all classes of antimicrobial agents increasingly complicates efforts to prevent and treat infections, especially in hospitalized patients. Of particular concern is the high and growing prevalence of methicillin-resistant *S. aureus* (MRSA): current hospital MRSA rates range from <1% in Sweden and Norway to >50% in Japan and the United States (5, 6). MRSA is a global problem with intermediate frequencies in hospitals in most of Europe, Australia, and several countries in Africa and South America (5). In recent years, MRSA also became an increasingly common cause of community-associated infections in persons without health care–associated risk factors (6).

Increased morbidity and mortality caused by MRSA have increased the financial burden on health care systems and concomitantly have induced strong interest in its evolutionary origins. Methicillin resistance is conferred by the penicillin-binding protein PBP2′, which catalyses the cross-linking of cell wall peptidoglycan and has a low affinity for β-lactam antibiotics. This enzyme is encoded by the *mecA* gene, which is located on a large fragment of mobile DNA designated "staphylococcal cassette chromosome" (SCC*mec*). SCC*mec* elements can integrate into the genome of staphylococci at a unique site near its origin of replication (7). Six different types of SCC*mec* have been identified on the basis of structural differences in both the recombinase gene complex and a second gene complex that includes *mecA* plus regulatory components (8, 9). Population biology studies based on multilocus enzyme electrophoresis and multilocus sequence typing (MLST) have indicated that MRSA have evolved multiple times through the acquisition of different types of SCC*mec* by distinct phylogenetic lineages of *S. aureus* (10). However, the true frequency of acquisition of SCC*mec* and other virulence determinants remains unknown, as do the factors that lead to the predominance of particular clones. Our ignorance on these matters is partially because *S. aureus* is so genetically homogeneous that MLST and other genotyping tools that currently are used for typing possess only limited discriminatory power. It already has been documented that individual MLST sequence types, including ST5, which is the subject of this report, can be heterogeneous with respect to antibiotic resistance patterns, *spa* type, SCC*mec* type, and gene content (11, 12). However, deeper understanding of the speed with which mobile elements and individual genes are imported and lost from *S. aureus* populations will require higher-resolution phylogenetic analyses than were possible until now.

An important, but still unresolved, question is whether the global occurrence of MRSA represents the geographic spread of individual epidemic clones. Only a few major clonal lineages (clonal complexes) dominate the population structure of contemporary MRSA, which provides circumstantial evidence for the massive global spread resulting from the selection of a small number of strains that have acquired resistance traits (7, 13). Alternatively, it remains possible that MRSA has arisen on multiple independent occasions through the local acquisition of SCC*mec* elements by methicillin-susceptible *S. aureus* (MSSA) strains and that MRSA clones appear ubiquitous only because

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they cannot be distinguished by currently used typing procedures. Interestingly, according to MLST, most MRSA clones that frequently are associated with nosocomial infections today also were predominant among MSSA isolates recovered during the 1960s from hospitals in Denmark (14) and more recently in England (15) and Belgium (16). Similar results were obtained in a carriage study from the United States by DNA macrorestriction analysis (17).

The most widespread type of sequence variation in bacterial genomes consists of SNPs, which result from point mutations. Genome-wide SNPs can be exploited to reveal the details of bacterial evolutionary history with great precision. For example, SNP-based approaches have been applied to investigate the phylogeny of several genetically monomorphic bacterial pathogens, including *Yersinia pestis* (18), *Mycobacterium tuberculosis* (19, 20), *Bacillus anthracis* (21), and *Salmonella* Typhi (22). In these young taxa, genomic variation is so rare that the investigation of their evolutionary history is technically challenging and, because of phylogenetic discovery bias, cannot be solved simply through the comparison of several random genomic sequences (19, 21, 23). To avoid discovery bias, it is important to elucidate the population structure indicated by neutral mutations in large, globally representative strain collections (22). Haplotypes that are representative of this population structure then can guide the rational choice of isolates for in-depth genome sequencing and resequencing (24). The results of such a rational approach can help to elucidate the biological and demographic processes that are responsible for the evolution of drug resistance or virulence and to identify genes and other genetic features, such as changes in genomic structure, that may be associated with these phenomena (25, 26).

Here, we report the global population structure of ST5 based on mutation discovery in *S. aureus* isolates from 22 countries in Europe, Africa, Asia, North America, South America, and Australia.

Results and Discussion

Nucleotide Diversity, Phylogeny, and Demographic History. We screened for sequence polymorphisms in 1.6% (46,483 bp) of the genome from each of 135 *S. aureus* isolates. To this end, 108 genome fragments of 450 bp, predominantly from housekeeping genes, were screened for heterozygosity against a reference strain through denaturing high-performance liquid chromatography (dHPLC)-based mutation discovery, and polymorphisms were elucidated by subsequent sequence analysis [supporting information (SI) Tables S1– S3]. The 135 strains all belonged to ST5 according to MLST and had been isolated in 22 countries from 6 continents. These analyses identified 156 bi-allelic polymorphisms (BiPs), corresponding to 1 BiP per 0.3 kilobases (Table S4). The 156 BiPs included 41 synonymous (silent) base substitutions in protein-coding genes, 92 non-synonymous substitutions, 13 substitutions in non-coding regions, and 10 deletions or insertions ranging in size from 1 to 52 bp (Table S4). The nucleotide diversity, π, was 0.00010 ± 0.00001 for coding regions and 0.00026 ± 0.00005 for non-coding regions (Table S1). The 156 BiPs were associated in 89 haplotypes among the 135 strains (Table S5).

The 156 BiPs were used to reconstruct the phylogeny of *S. aureus* ST5. Twenty-five BiPs were informative for maximal parsimony analyses because each was found in >1 haplotype. A minimum spanning tree of the 89 haplotypes (Fig. 1) has a radial branch structure and is rooted at a single ancestral node, haplotype H1, whose character states for all 156 BiPs are identical to those from all 7 genome sequences that are available from other clonal complexes of *S. aureus* (Fig. 1*a*). Similar to prior results in Typhi (22) and *M. tuberculosis* (20), the level of homoplasy in this tree is extremely low (homoplasy index, 0.04), resulting in an almost unique phylogeny (details in *SI Text*). Furthermore, most of the step-wise accumulations of single BiPs from the ancestral node are represented by 1 of the 135 extant isolates. This finding indicates long persistence of haplotypes through evolutionary time (20, 22). Based on an *Escherichia coli* molecular clock rate (27), we estimate that the most recent common ancestor of the ST5 radiation existed $\approx$ 2300 years ago and that the population size has expanded over the last 1000 years (Figs. S2–S4; details in *SI Text*).

Phylogeography. Within the minimum spanning tree, most isolates are clustered according to geographic origin, with only few exceptions that may reflect recent migration events (Figs. 1*A* and 2, and Table S6). For example, lineage G encompasses all 22 isolates from East Asia, including South Korea, Taiwan, Japan, and Hong Kong, but only 2 isolates from outside East Asia (Belgium, Switzerland). Similarly, all 19 isolates from South Africa and the sole isolate from Kenya are in lineage D; 6 of the 9 isolates from North America are in lineage K; and all 4 isolates in lineage J are from Australia (Fig. 2). Isolates of European origin appear to be more diverse and are found in multiple lineages (Fig. 1*A*), but the difference between Europe and other areas may represent a sampling bias resulting from more extensive coverage, because 51% of the isolates tested were from Europe. In addition to continent-specific lineages, our data suggest that sublineages exist that are even more strongly confined geographically (Fig. 1*A*). For example, isolates from Japan, Taiwan, Hong Kong, Poland, Israel, and South Africa, some of which were collected over a span of decades and in different laboratories, are each associated in country-specific haplotypes, sets of related haplotypes, or lineages (Fig. 1*A*). The 15 isolates in lineage L, consisting of haplotype H77 plus its descendants, include strains that were submitted to the German National Reference Center >7 years by 13 different clinical laboratories in Germany, plus 2 isolates from the neighboring countries Austria and Netherlands (Fig. 1*A*). These observations indicate that much of the population structure of ST5 is local. Haplotypes and lineages within *S. aureus* thus are largely endemic within individual geographical areas and spread to other countries or continents only on rare occasions.

Geographic localization may reflect the fact that *S. aureus* is primarily an endemic commensal that colonizes the human nasal mucous membranes (17). Little is known about the determinants of asymptomatic carriage, but longitudinal studies have shown that colonization may persist for years in some individuals (28). Hence, the geographic distribution patterns and population structure observed for *S. aureus* may be determined primarily through nasal carriage rather than through occasional infectious disease. Spread of *S. aureus* clones associated with patient transfers between different hospitals has been observed (29). However, such patient transfers are likely to be local and only rarely extend over long distances or across national borders. Person-to-person transmission requires close contact, restricting international dispersal even in the era of abundant air travel.

Correlation of Toxin Gene Complement with Haplotype Lineages. Our interpretation that the population structure of *S. aureus* reflects an endemic commensal lifestyle raises the possibility that virulence may be associated with different population dynamics. Virulence may be important for facilitating host-to-host transmission (30), potentially leading to selective pressures for the expression of particular virulence determinants. Hundreds of genes are suspected of contributing to *S. aureus* pathogenesis. However, little is known about the effects that individual factors may have on the bacterium's propensity to cause disease, aside from a few toxins implicated in specific toxin-mediated diseases (31). Toxin genes are variably present within *S. aureus* isolates, possibly because they are located on prophages and genomic islands, which are thought to be mobile vectors of horizontal

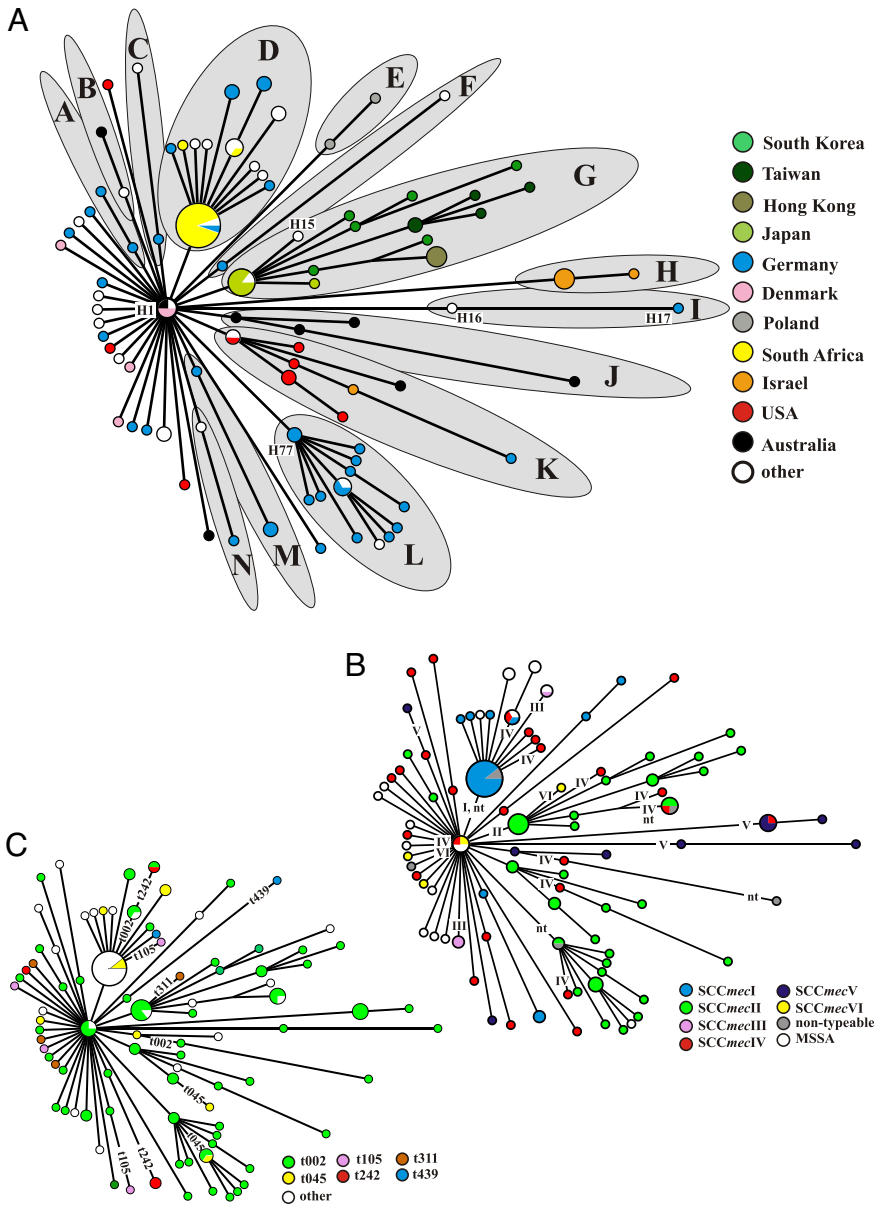

Fig. 1. Minimum spanning tree based on 156 BiPs discovered in 46,483 bps of DNA from each of 135 *S. aureus* ST5 isolates from a global collection. Circle size is proportional to haplotype frequency, and line length is proportional to the number of mutational steps between haplotypes. (*A*) Colors indicate the countries of origin of the isolates. Fourteen lineages that consist of at least 2 haplotypes are labeled ''A'' through ''N.'' Numbers of some haplotypes referred to in the text are indicated. (*B*) Colors indicate the type of SCC*mec* in each of the MRSA isolates. SCC*mec* elements that are non-typeable on the basis of current PCR schemes probably represent SCC*mec* variants, because they displayed recombinase gene (*ccrB*) sequences that were < 93% homologous to published *ccrB* sequences. Roman numerals indicate events of SCC*mec* acquisition that explain the observed distribution of SCC*mec* elements in the most parsimonious way. The actual number of SCC*mec* acquisitions may be higher. (*C*) Colors indicate homoplasies among *spa* sequences. The emergence of *spa* sequences that already exist elsewhere in the tree is labeled with text. This tree provides a parsimonious explanation for the homoplasies, but alternative scenarios exist that invoke the same or larger numbers of homoplasies for the observed distribution of *spa* sequences. For example, the interpretation shown here invokes the repeated evolution of t002 through the acquisition of 3 contiguous sequence repeats, whereas an alternative explanation is that t045 evolved through the loss of those 3 repeats.

transfer. We therefore were prepared to detect poor correlations, if any, between the presence of toxin genes and phylogenetic descent. Most of the 135 strains lacked the 7 genes that we tested by dedicated PCR reactions (Fig. S1). However, in that minority of strains that did possess toxin genes, these genes seem to be maintained within a lineage once they have been acquired and are lost only rarely. For example, both haplotypes of lineages E, I, and N are uniform in relation to the *seb*, *tst*, *lukS/F*, and *sea* genes; and the *tst* gene was present in all 22 isolates from East Asia within lineage G, with 1 exception in which it may have been lost as a secondary event. Thus, these data suggest that the acquisition of virulence determinants does not necessarily lead to global spread but rather reflects local phylogeographic patterns similar to those that apply for neutral genes.

***spa* Typing Reflects Homoplasies.** Sequence analysis of the tandem repeat region of the *spa* gene is widely applied for typing *S. aureus* isolates, partly because sequencing this single genetic locus is less costly than MLST (32). Each unique repeat region sequence is assigned to a distinct *spa* type. However, mapping of 25 *spa* types (Table S2) onto the BiP-based minimum spanning tree revealed multiple *spa* sequences within ST5 whose patterns

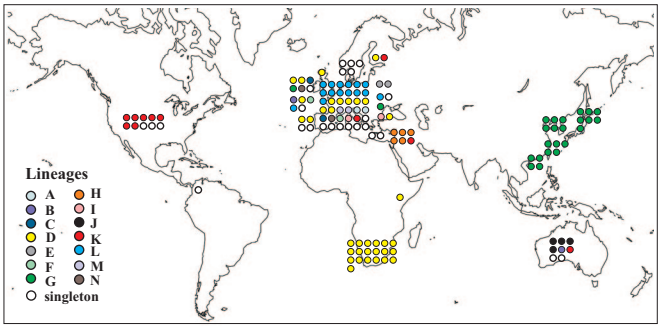

Fig. 2. Geographic distribution of phylogenetic lineages "A" through "N" from Fig. 1*A*. Colors indicate the phylogenetic affiliations of 135 isolates.

seemed to reflect homoplasies (Fig. 1*C*). Of the 10 *spa* types that were each represented by at least 2 *S. aureus* isolates, 6 were associated with 2 or more unrelated haplotypes and/or distinct lineages (Fig. 1*C*). In these cases, the sequence identity between *spa* types in unrelated haplotypes probably reflects the result of repeated convergent evolution of *spa* sequences on multiple occasions. Alternatively, the haplotypes may have converged through homoplasies, but this alternative is less parsimonious because each such homoplasy would require multiple rare BiPs that are scattered around the genome to have converged by mutation or recombination. This finding has important implications for the use of *spa* sequences as a primary form of staphylococcal typing in many laboratories. Perhaps most importantly, *spa* sequences can suggest misleadingly that individual clones are spreading globally. For example, *spa* type t045 was found in isolates from Germany, Belgium, South Africa, Kenya, the United States, Colombia, and Australia (Fig. 1*C* and Table S2). However, these isolates are affiliated with at least 3 separate haplotype lineages that are geographically confined (Fig. 1 *A* and *C*) and do not represent a single clone. Similarly, *spa* types t105 and t242 in Germany and Austria are each associated with three independent lineages (Fig. 1*C*).

spa Sequences are so variable because they represent tandem sequence repeats that are subject to frequent polymerase staggering during DNA replication and therefore evolve more quickly than most protein-coding regions of the *S. aureus* genome. For example, 4 of the 6 *spa* sequences with homoplasies (t105, t311, t439, and t045) correspond to deletions of 1 to 3 repeats at central positions within the 10 repeat units that are present in the presumed ancestral type, t002. Considering the low number of repeats available to generate variation, the repeated loss of the same repeats through deletion is likely, even from a neutral perspective. It also is possible that variant *spa* types are selected by host's immune system because the *spa* gene product is a secreted virulence factor (33). Generally, if *spa* typing is to be reliable and informative, it might be advisable to embed such highly variable genomic loci as a second-level marker within a progressive, hierarchical approach to bacterial genotyping in which the top level is based on canonical SNPs that are reliable indicators of key phylogenetic branches (34).

Evolutionary History of Methicillin Resistance. SCC*mec* is large (21–67 kb) and is difficult to transfer between strains in the laboratory (35). As a result, until recently, MRSA was thought to have arisen via global dissemination after a limited number of imports of SCC*mec* (7, 13). However, SCC*mec* does not seem to differentiate rapidly, because structural variants are maintained stably (8) and genetic recombination between SCC*mec* elements is very rare (36). Therefore, the existence of different SCC*mec* variant types suggests SCC*mec* elements have been imported on multiple, independent occasions (8, 10, 36). Recent comparisons of structural diversity within SCC*mec* versus strain differentiation of housekeeping genes (MLST) and surface protein genes have resulted in the interpretation that SCC*mec* has been imported on at least 20 independent occasions into different phylogenetic lineages of susceptible *S. aureus* (8, 10). The data obtained here indicate that this estimate is still at least 1 order of magnitude too low, because at least 23 SCC*mec* acquisition events seem to have occurred within ST5 alone (Roman numerals in Fig. 1*B*). ST5 is part of a higher-level group designated "clonal complex CC5," which includes multiple descendants of ST5 that display nucleotide variation at 1 or several MLST loci. We note, however, that non-ST5 isolates from CC5 are not necessarily any more divergent from the common ancestor than average (authors' unpublished full-genome sequence data). MRSA are common not only throughout CC5 but also in 5 other common clonal complexes of *S. aureus* (CC1, 8, 22, 30, 45) and in a number of less prevalent clonal complexes (including CC59, 78, 80, 89, 97, 398; see www.mlst.net). Thus, we anticipate that many hundreds of independent imports of SCC*mec* will be identified with time.

Our results further suggest that the international dissemination of MRSA is rare because many of the acquisitions of SCC*mec* were at terminal tips of the minimum spanning tree that had not disseminated (Fig. 1*B*). Even the presence of SCC*mec* throughout most isolates of individual lineages argues against dissemination because these lineages were themselves geographically restricted. These observations seem to be counterintuitive, because it might have been expected that the selective pressure imposed by antibiotic usage would select for the transmission of MRSA clones once they had arisen through horizontal gene transfer. However, MRSA apparently have arisen locally on so many occasions that extensive transmission probably will be successful only when an individual MRSA clone is more fit than the preexisting MRSA clones in other areas (37), and this greater fitness seems to be a rare event in *S. aureus*. Future work will show if similar distribution patterns also apply to other MRSA clones. Our interpretation that MRSA has emerged frequently predicts that the MRSA haplotypes within a local area also should be present in MSSA from that area. That interpretation cannot be tested with the currently available data, because only few MSSA strains were tested here. However, the BiPs reported in this study provide the tools to test this interpretation in future studies. A further prediction from our results is that the sources of SCC*mec* also should be present in the same local areas as the MRSA strains; this prediction would justify searching for such donors among other MRSA or methicillin-resistant coagulase-negative staphylococci (*S. epidermidis* and several related species) that commonly co-colonize individual hosts together with *S. aureus* (38).

We note that our conclusions regarding frequent local evolution of MRSA are reminiscent of international classification codes used during the 1990s, which subdivided MRSA strains within ST5 into regional groupings designated "Japan," "New York," "Rheinhessen" (Germany), "Paediatric" (Portugal), and "EMRSA-3'" (England) (39) on the basis of macrorestriction DNA analysis. These groupings are no longer widely used because they were not supported by MLST and because of technological problems associated with macrorestriction assays. In the case of ST5, some isolates representing different macrorestriction-based pulsotypes were affiliated with distinct haplotypes and haplotype lineages (Table S7), whereas other isolates from distinct lineages displayed indistinguishable macrorestriction patterns (Table S7). Therefore, at least some of the macrorestriction groupings may represent distinct MRSA clones that simply could not be resolved by MLST.

On the basis of their similar macrorestriction patterns, MLST, and antibiograms, close evolutionary relationships have been postulated between contemporary Paediatric and New York

MRSA and MRSA isolates recovered between 1957 and 1973 from hospitals in Denmark (13). Because of these correlations and because Denmark was among the first countries in which MRSA was discovered early in the 1960s, it was hypothesized further that MRSA emerged in Denmark and then spread globally (13). Five MSSA isolates from the historical Danish MSSA collection were included in the present study (Table S2). All 5 Danish strains are related to each other. Isolates E1044 and E6030, isolated in 1960 and 1972, are in the ancestral haplotype H1 from which all modern MRSA haplotypes are derived. These results are indeed consistent with a Danish origin of the Paediatric and New York strains, although genomic resequencing and analyses of larger numbers of isolates will be needed for a definitive reconstruction of the origins of MRSA strains within ST5. The 3 other Danish MSSA strains (E3001, E1293, and E323) apparently have not contributed to the origin of modern MRSA because they are on derived lineages that lack MRSA strains (Fig. 1*A*).

In a second preliminary analysis, we also examined 3 health care–associated MRSA strains from Germany, Slovenia, and Switzerland. These strains were assigned to haplotypes H16, H17, and H15, 2 of which are on a lineage that lacks hospital isolates (Fig. 1*A*). This observation reinforces the notion that health care–associated MRSA strains may be of distinct evolutionary origins from hospital strains (6), as previously suggested by MLST and/or DNA macrorestriction analysis in combination with SCC*mec* typing and toxin gene content (11).

Conclusions

We have investigated the evolutionary history of MRSA within ST5 with a globally representative collection of 135 methicillin-resistant and methicillin-susceptible isolates. The analysis was based on mutation discovery within 108 gene loci that span 46 kb. The polymorphisms described here can be used immediately for medium-resolution epidemiological investigations (40, 41). High-resolution epidemiology must await genomic resequencing of multiple isolates that can be chosen rationally to represent the phylogenetic framework of the haplotypes as described here (24).

The geographical distribution of haplotypes within ST5 showed strong phylogeographic associations, similar to the genetically monomorphic *M. tuberculosis* (20) but unlike *Salmonella* Typhi (22). Our data indicate that SCC*mec* has been imported at least an order of magnitude more frequently than previously appreciated. The data also provide strong evidence that MRSA have emerged on numerous occasions in distinct locations and that geographic dispersal is limited. This conclusion challenges a general perception that only few MRSA strains exist and that these epidemic clones have spread globally.

Materials and Methods

Bacterial Isolates. Sources and properties of 135 isolates of *S. aureus* are listed in Table S2. All these isolates had been typed previously by MLST as sequence type ST5. MLST, *spa* typing, and the characterization of SCC*mec* elements were performed as described previously (32). Recombinase genes (*ccrB*) from SCC*mec* elements were PCR-amplified and sequenced as described previously (36). PCR primers for amplification of toxin genes *sea*, *seb*, *sec*, *tst*, and *lukS/F* are listed in Table S8. Isolates from 22 countries in Africa, Europe, Asia, Australia, North America, and South America were included, to encompass global diversity (Table S2).

Genetic Loci, PCR Primers, and Polymorphisms. The genetic loci investigated are listed in Table S3. As indicated, their genomic positions are scattered around the genome of reference strain N315. The PCR primers used to amplify fragments of these genetic loci are given in Table S3. The polymorphisms discovered and their properties are given in Table S9.

Mutation Discovery by dHPLC. Mutation discovery was performed as described previously for *Salmonella* Typhi (22). Bacterial isolates were compared with the reference strain N315, for which the genome had been fully sequenced previously (42). PCR-amplified gene fragments were analyzed by dHPLC (WaveR Nucleic Acid Fragment Analysis System, Transgenomic) at the temperatures given in Table S3. Polymorphic PCR products were sequenced subsequently from both ends by using the PCR primers.

Data Analysis. A minimum spanning tree based on 156 BiPs from 135 isolates was constructed with Bionumerics 5.1 (Applied Maths NV, Sint-Martens-Latem, Belgium). The ancestral node was determined by comparison with all available genome sequences from *S. aureus* strains affiliated with different clonal complexes, including COL (GenBank accession number CP000046, sequence type ST250), NCTC8325 (NC007795, ST8), USA300 (CP000255, ST8), Newman (AP009351, ST254), RF122 (AJ938182, ST151), MW2 (BA000033, ST1), and MSSA476 (BX571857, ST1). Details about analyses performed on concatenated sequences from the investigated loci are in SI Text.

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