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Clonal Analysis of Methicillin-Resistant *Staphylococcus aureus* Strains from Intercontinental Sources: Association of the *mec* Gene with Divergent Phylogenetic Lineages Implies Dissemination by Horizontal Transfer and Recombination

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Genetic relationships among 254 isolates of *Staphylococcus aureus* resistant to methicillin recovered between 1961 and 1992 from nine countries on four continents were determined by analyzing electrophoretically demonstrable allelic variation at 15 chromosomal enzyme loci. Fifteen distinctive electrophoretic types, marking clones, were identified. The *mec* gene is harbored by many divergent phylogenetic lineages representing a large portion of the breadth of chromosomal diversity in the species, a result that is interpreted as evidence that multiple episodes of horizontal transfer and recombination have contributed to the spread of this resistance determinant in natural populations. Isolates recovered in the United Kingdom, Denmark, Switzerland, Egypt, and Uganda in the 1960s are of a single multilocus enzyme genotype and probably are progeny of an ancestral methicillin-resistant clone. There is geographic variation in the frequency of recovery of the common methicillin-resistant clones, an observation that may in part explain reported regional differences in natural history correlates of resistant organisms.

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were first reported in 1961 (18), very soon after methicillin entered clinical use, and within a few years, outbreaks of MRSA were recorded in the United Kingdom and elsewhere in Europe. In the mid-1970s, MRSA was recognized as a significant problem in the United States, with the documentation of several outbreaks in tertiary-care teaching hospitals (1, 6, 10, 35). These resistant organisms are now commonly recovered in virtually every large hospital in the United States, and, in addition, MRSA isolates have achieved global distribution (15). MRSA also have become a significant infection control problem in nursing homes and other chronic-care facilities (6, 44).

Intrinsic methicillin resistance is due to the expression of an altered penicillin-binding protein (PBP 2a) (17) that is absent in susceptible isolates. The protein is encoded by a chromosomal gene (*mec*) (24) that apparently originated as a consequence of a recombinational event fusing about 300 bp of a staphylococcal β-lactamase gene and part of a gene encoding a penicillin-binding protein from an unknown donor organism, perhaps *Escherichia coli* (41, 42). Expression of the *mec* gene is constitutive in many strains, whereas in others it is inducible (47, 49), perhaps by a mechanism similar to that regulating β-lactamase synthesis. Evidence has also been presented that other chromosomal loci may be involved in regulating *mec* expression (2, 3).

Many typing schemes have been employed to study MRSA spread in epidemiologic investigations. The techniques used to examine these isolates include bacteriophage typing (1, 11, 36), antibiotic susceptibility testing (11), electrophoretic profiling of whole-cell proteins (12, 45) and plasmids (1, 14, 30), and, more recently, profiling of plasmid and chromosomal restriction fragment length polymorphisms (5, 9, 13, 19, 23, 31, 51), pulsed-field gel electrophoresis (33, 34), ribotyping (4, 16, 32), and others (7–9, 20, 25). These techniques, used alone or in combination, have proven useful to address problems of strain dissemination.

However, despite intensive study of MRSA, certain basic questions remain unanswered. First, it is unknown exactly how many chromosomal genotypes commonly harbor the *mec* gene nor are the genetic relationships among these chromosomal lineages known. Second, it is unknown whether the *mec* gene is found in association with the breadth of phylogenetic lines of the species *S. aureus* or whether MRSA isolates represent a restricted subset of lineages. Third, the genetic relationships among MRSA strains circulating in different geographic areas are poorly understood. The purpose of this study was to address these and other questions through the use of multilocus enzyme electrophoresis and other molecular population genetic techniques.

MATERIALS AND METHODS

Bacterial isolates. We examined a total of 254 isolates of MRSA, including 227 isolates from 10 states (New York, Pennsylvania, Rhode Island, Massachusetts, New Jersey, Wisconsin, Illinois, Kentucky, South Carolina, and Texas), 11 isolates from five provinces in Canada (Nova Scotia, Quebec, Ontario, Manitoba, and Alberta), 5 isolates from Ireland, 1 isolate from Australia, 3 isolates from Denmark, 1 isolate from Egypt, 1 isolate from Uganda, 4 isolates from the United Kingdom, and 1 isolate from Switzerland. Isolates were recovered between 1961 and 1992. On the basis of a characterization of a larger MRSA sample by restriction fragment length polymorphism typing with several genes, strain MSA 3418 from Australia is representative of the endemic MRSA organisms circulating in that country (19a). A partial list of the strains is presented in Table 1, and a complete list is available upon request.

The great majority of the isolates were recovered from

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TABLE 1. ET, collection locality, and date of isolation for 78 MRSA strains

ET	Isolate	Locality	Date
1	MSA 1560	Rhode Island	1980s
	MSA 1567	Rhode Island	1980s
2[a]	MSA 3399	Long Island, N.Y.	1980s
	MSA 3401	Manhattan, N.Y.	1980s
	MSA 3422	Brooklyn, N.Y.	1980s
	MSA 3434	Bronxville, N.Y.	1980s
	MSA 3440	Manhattan, N.Y.	1980s
	MSA 3444	Brooklyn, N.Y.	1980s
	MSA 1887	Rochester, N.Y.	1980s
	MSA 3423	New Jersey	1980s
	MSA 3425	New Jersey	1980s
	MSA 851	Rhode Island	1980s
	MSA 1558	Massachusetts	1980s
	MSA 1554	Kentucky	1980s
	MSA 1840	Chicago, Ill.	1980s
	MSA 3424	Wisconsin	1980s
	MSA 3398	Winnipeg, Canada	1980s
	MSA 3405	Edmonton, Canada	1980s
	MSA 3400	Dublin, Ireland	1990
3	MSA 829	Rhode Island	1980s
4	MSA 1902	Rochester, N.Y.	1986
	MSA 1591	Rhode Island	1980s
5	MSA 847	Providence, R.I.	1980s
	MSA 3432	New York, N.Y.	1980s
6	MSA 3414	Toronto, Canada	1987
7	MSA 3402	Manhattan, N.Y.	1978
	MSA 3415	Bronxville, N.Y.	1980s
	MSA 3416	Brooklyn, N.Y.	1980s
	MSA 3419	Manhattan, N.Y.	1980s
	MSA 3420	Yonkers, N.Y.	1980s
	MSA 3433	New Jersey	1980s
	MSA 3435	New Jersey	1980s
	MSA 3403	Manitoba, Canada	1980s
	MSA 3404	Montreal, Canada	1980s
	MSA 3428	Dublin, Ireland	1980s
	MSA 3418	Australia	1980s
8	MSA 1600	South Carolina	1980s
	MSA 1601	Rhode Island	1980s
	MSA 1602	Rhode Island	1980s
	MSA 1603	Rhode Island	1980s
9[b]	MSA 3410	London, England	1960s
	MSA 3437	London, England	1960s
	MSA 3443	London, England	1964
	MSA 3409	Dublin, Ireland	1980s
	MSA 3426	Dublin, Ireland	1980s
	MSA 3427	Dublin, Ireland	1980s
	MSA 3429	Denmark	1960s
	MSA 3430	Denmark	1960s
	MSA 3431	Denmark	1960s
	MSA 3441	Uganda	1966
	MSA 3442	Geneva, Switzerland	1962
	MSA 3445	Cairo, Egypt	1961
	MSA 3411	London, Canada	1980s
	MSA 3421	Toronto, Canada	1986
	MSA 1940	Pennsylvania	1980s
	MSA 868	Dallas, Tex.	1980s
	MSA 3439	CDC[c]	Unknown
10	MSA 1594	Rhode Island	1980s
	MSA 3397	Edmonton, Canada	1980s
11	MSA3438	London, England	1960s
12	MSA1588	Providence, R.I.	1980s
	MSA1589	Providence, R.I.	1980s
13	MSA 861	Rhode Island	1980s
14[d]	MSA 817	Rhode Island	1980s
	MSA 830	Rhode Island	1980s
	MSA1906	Rochester, N.Y.	1985
	MSA1909	Rochester, N.Y.	1985
	MSA1927	Rochester, N.Y.	1986
15	MSA3408	Brooklyn, N.Y.	1980s
	MSA3412	Brooklyn, N.Y.	1980s
	MSA3436	New York	1980s
	MSA1891	Rochester, N.Y.	1980s
	MSA 831	Rhode Island	1980s
	MSA1878	Chicago, Ill.	1980s
	MSA3406	Toronto, Canada	1980s
	MSA3407	Bronxville, N.Y.	1980s
	MSA3413	Halifax, Canada	1980s
	MSA3417	Toronto, Canada	1986

[a] Fifty isolates from four hospitals in Rhode Island; 49 isolates from one hospital in Rochester, N.Y.; 34 isolates from one hospital in Chicago, Ill.; 4 isolates from one hospital in Boston, Mass.; 2 isolates from one hospital in Kentucky.

[b] Forty-seven isolates from one hospital in Dallas, Tex.; 3 isolates from one hospital in Pittsburgh, Pa.

[c] CDC, Centers for Disease Control, Atlanta, Ga.

[d] Eleven isolates from three hospitals in Providence, R.I.; 3 isolates from one hospital in Rochester, N.Y.

patients with clinically significant disease episodes, and a wide range of infection types were represented in the sample. All isolates were identified as MRSA on the basis of conventional antimicrobial susceptibility testing procedures routinely employed in clinical microbiology laboratories (29). Forty-nine of the isolates, including all organisms recovered from foreign sources, have been examined for hybridization with a probe for the *mec* gene after restriction fragment length polymorphism genomic profiling and were kindly supplied by B. N. Kreiswirth, Public Health Research Institute, New York, N.Y. These latter strains were analyzed for multilocus enzyme genotype in a blinded fashion. The sample includes 165 isolates from the United States that were partially characterized in previous studies (28, 30) of genetic relationships among MRSA isolates; 8 of the 173 organisms of the original sample were omitted from the present analysis because they were found to be incorrectly classified as MRSA.

Culturing of bacteria and electrophoresis of enzymes. Each isolate was grown in 150 ml of tryptic soy broth (Difco Laboratories) overnight at 37°C on an orbital shaker (250 rpm) and harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. After suspension in 1.5 to 2.0 ml of 50 mM Tris-HCl buffer containing 5 mM EDTA (pH 7.5), lysostaphin (Sigma) was added to a final concentration of 100 μg/ml, and the cells were incubated at 37 to 40°C for 45 min in a water bath. The bacteria were sonicated with a Branson model 200 sonifier-cell disruptor equipped with a microtip for 30 s at 50% pulse, with dry ice-methanol cooling, and were centrifuged at 20,000 × *g* for 20 min at 4°C. The clear supernatant was electrophoresed immediately or stored at −80°C.

Lysates were electrophoresed on starch gels and selec-

TABLE 2. Allele profiles at 15 enzyme loci in 15 ETs of MRSA strains

ET	Reference isolate[a]	No. of isolates	Allele at indicated enzyme locus[b]													
			ACO	PGI	CAK	G6P	M1P	6PG	GLD	NSP	CAT	EST	LD1	LD2	ADH	IPO	SHK
1	1560	2	5	5	5	5	5	5	5	7	5	5	5	8	5	5	5
2	3399	151	5	5	5	5	5	5	5	5	5	5	5	8	5	5	5
3	829	1	5	5	5	5	5	5	5	4	5	7	5	8	5	5	5
4	1902	2	5	7	5	5	5	5	5	5	5	3	5	8	5	5	5
5	847	2	5	7	5	5	4	5	5	5	5	3	5	8	5	5	5
6	3414	1	6	5	5	5	4	5	5	5	5	6	5	8	5	5	5
7	3402	11	5	5	5	5	4	5	5	5	5	6	5	8	5	5	5
8	1600	4	5	5	5	5	4	5	5	5	5	3	5	8	7	5	5
9	3410	50	5	5	5	5	4	5	5	5	5	3	5	8	5	5	5
10	3397	2	5	5	5	7	4	5	5	5	5	3	5	8	7	5	5
11	3438	1	5	5	5	5	4	5	5	5	6	3	5	8	5	5	7
12	1588	2	5	5	7	5	4	5	5	5	5	9	5	8	5	6	5
13	861	1	5	5	5	5	5	4	5	5	5	7	5	10	5	5	3
14	817	14	5	5	5	5	5	5	5	5	5	7	5	10	5	5	3
15	3408	10	6	5	5	5	5	5	5	5	6	6	5	5	5	5	5

[a] Musser *S. aureus* strain number.

[b] ACO, aconitase; PGI, phosphoglucose isomerase; CAK, carbamylate kinase; G6P, glucose-6-phosphate dehydrogenase; M1P, mannitol-1-phosphate dehydrogenase; 6PG, 6-phosphogluconate dehydrogenase; GLD, glutamate dehydrogenase; NSP, nucleoside phosphorylase; CAT, catalase; EST, esterase; LD1, lactate dehydrogenase-1; LD2, lactate dehydrogenase-2; ADH, alcohol dehydrogenase; IPO, indophenol oxidase; SHK, shikimate dehydrogenase.

tively stained for 15 metabolic enzymes (27, 38). The enzymes studied were aconitase, phosphoglucose isomerase, carbamylate kinase, glucose-6-phosphate dehydrogenase, mannitol-1-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutamate dehydrogenase, nucleoside phosphorylase, catalase, esterase, lactate dehydrogenase-1, lactate dehydrogenase-2, alcohol dehydrogenase, indophenol oxidase, and shikimate dehydrogenase.

Distinctive electromorphs (mobility variants) of each enzyme, numbered in order of decreasing rate of anodal migration, were equated with alleles at the corresponding structural gene locus. Because virtually all isolates, including some that lacked detectable plasmids, showed activity for all 15 enzymes, we presume that the corresponding structural gene loci are located on the chromosome.

Each isolate was characterized by its combination of alleles at the 15 enzyme loci, and distinctive combinations of electromorphs, corresponding to unique multilocus enzyme genotypes, were designated as electrophoretic types (ETs).

Statistical analysis. Single-locus genetic diversity among ETs and mean diversity per locus were calculated as previously described (38).

Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which different alleles were represented (mismatches), and clustering of ETs was performed from a matrix of genetic distances by the average linkage method (38).

RESULTS

Genetic and genotypic diversity. In the collection of MRSA isolates examined, 13 of the 15 enzyme loci assayed were polymorphic for alleles encoding electrophoretically distinguishable variant polypeptides and 2 loci (glutamate dehydrogenase and lactate dehydrogenase-1) were monomorphic (Table 2). A total of 15 distinctive ETs were identified (Table 2), many of which differed at only one or a few loci. Mean genetic diversity per locus (H) among the ETs was 0.255, and there was an average of 2.3 alleles per locus. The esterase locus was the most polymorphic, with five alleles represented in our sample.

Genetic relationships among multilocus genotypes. The dendrogram in Fig. 1 summarizes estimates of the genetic relationships of the 15 ETs. The smallest observed genetic distance (0.07) between ETs corresponds to a single-locus difference, and the largest distance (0.35) corresponds to differences at 6 of the 15 loci. At a distance of 0.20, there were six lineages or clusters, designated A through F; clusters A, B, and E were composed of 3, 7, and 2 ETs, respectively, and the C, D, and F lineages were each represented by a single ET. ETs of clusters A and B differed, on average, at two and three loci, respectively; lineage D was somewhat more divergent from clusters A and B and lineage C, with a mean distance of 0.28. Cluster F diverged from lines A to E at a distance of 0.35, reflecting the occurrence of dissimilar alleles at an average of six loci.

In summary, multilocus enzyme electrophoresis identified six divisions of MRSA isolates: (i) cluster A (ETs 1 through 3), (ii) cluster B (ETs 4 through 10), (iii) lineage C (ET 11), (iv) lineage D (ET 12), (v) cluster E (ETs 13 and 14), and (vi) lineage F (ET 15).

Geographic distribution of ETs. Individual ETs may have wide, even, intercontinental distribution. For example, isolates of ET 2 in our sample were recovered from patients in the United States (Rhode Island, Massachusetts, New York, New Jersey, Kentucky, Illinois, Wisconsin, and Texas), Canada (Manitoba and Alberta), and Ireland, and isolates of ET 9 were from the United States (Pennsylvania, Illinois, and Texas), Canada (Ontario), the United Kingdom, Ireland, Denmark, Switzerland, Egypt, and Uganda. Isolates of ET 15 were cultured only from patients in North America.

Temporal recovery of clones. With the single exception of MSA 3438 (ET 12), all isolates recovered in the 1960s were ET 9.

DISCUSSION

Nature of the sample studied. Because MRSA isolates from the United States and Canada were obtained from several geographic regions and diverse types of infections, the samples from these countries are believed to be representative of the populations of pathogenic strains resistant to methicillin. We acknowledge that some of the strains examined from U.S. sources were recovered from patients admit-

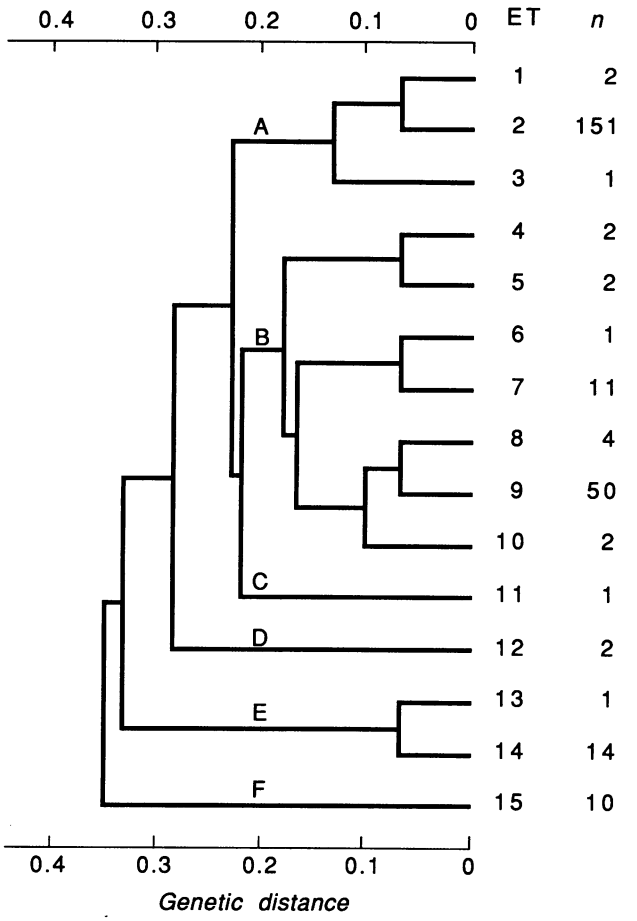

FIG. 1. Dendrogram showing estimates of genetic relationships of 15 ETs of MRSA isolates based on allele profiles at 15 enzyme loci. The dendrogram was generated from a matrix of genetic distances between pairs of ETs by the average linkage method (38). *n*, number of isolates assigned to each ET.

ted to the same hospitals and therefore probably represent redundant sampling as a consequence of nosocomial transmission. Our sample from countries other than the United States and Canada is limited in both size and temporal breadth, and, therefore, conclusions drawn from the study of the isolates in that sample must take this factor into consideration.

Primary observation. By using allelic enzyme variants revealed by starch gel electrophoresis to index levels of genetic diversity and to estimate phylogenetic relationships among MRSA strains, we have found that the *mec* gene commonly occurs in association with several distinct multilocus enzyme genotypes. Because evolutionary convergence to the same multilocus enzyme genotype is highly unlikely (39), the most parsimonious explanation for the repeated recovery of isolates of the same ET is that they share lineal descent from a common precursor cell, that is, the isolates represent a clone.

On the basis of identification of a single multilocus enzyme genotype among organisms recovered in the United Kingdom, Denmark, Switzerland, Uganda, and Cairo, soon after the widespread introduction of methicillin into clinical use in the 1960s, we favor the idea that MRSA isolates recovered from these localities are the progeny of a single ancestral cell that acquired the *mec* determinant, perhaps recently. The idea of a clonal origin for European MRSA strains recovered in the 1960s and early 1970s is not new (21).

We also hypothesize that the association of *mec* with additional divergent clonal lineages has occurred as a consequence of horizontal transfer and recombination of this resistance gene to methicillin-sensitive precursor cells. If this is the case, several transfer events are required to explain the pattern of *mec* distribution identified by our analysis. Three lines of evidence are cited supporting the idea of horizontal transfer and recombination of *mec* rather than chromosomal divergence following resistance acquisition by a single precursor cell, such as an organism assigned to ET 9. First, susceptible isolates of ET 2, ET 7, and ET 15 are abundantly represented in a collection (28) of about 2,500 *S. aureus* isolates from widespread localities and from several human infection types and bovine mastitis (unpublished data). Second, isolates assigned to ET 15 are sufficiently differentiated from organisms of the other ETs to suggest that divergence of these resistance clones from a single resistant genotype arising in the 1960s is unlikely, especially inasmuch as evidence exists that alleles of metabolic enzyme loci are selectively neutral (50) and therefore do not endow a strong fitness advantage. Third, evidence has been presented that *mec* is carried by a genetic element that may be mobile in natural populations (43, 46, 48). As discussed previously (28), in theory the occurrence of *mec* in association with distinct chromosomal lineages could be explained by several origins of this resistance gene. However, comparison of the *mec* sequence from two independent MRSA strains and an isolate of methicillin-resistant *Staphylococcus epidermidis* identified only nominal allelic diversity (37), a result suggesting a single origin for *mec*. The lack of sufficient samples of contemporary strains from foreign sources limits the conclusions that can be drawn regarding the number of multilocus enzyme genotypes currently circulating in Europe or elsewhere. In this regard, we note that Branger and Goullet recently examined esterase electrophoretic polymorphism in a sample of 136 MRSA isolates cultured from patients in 18 countries, including 10 in Europe, and concluded that the methicillin-resistant phenotype occurs in association with genetically diverse strains (8). These investigators also suggested that, on average, European MRSA are genetically different from those organisms recovered in the United States. Our data support their ideas in large part.

Use of multilocus enzyme electrophoresis for molecular epidemiologic studies of MRSA strains. Despite evidence to the contrary (28, 30), the claim was recently made (4, 26) that the utility of enzyme electrophoresis for discriminating MRSA strains is unclear. The data presented here and elsewhere (28, 30) demonstrate that this technique resolves sufficient polymorphisms among MRSA strains to be of use in addressing certain questions of routine interest in local bacteriologic epidemiology. It is also clear, on the basis of data presented previously (30), that other techniques such as plasmid profiling offer additional resolving power useful for the examination of fine-structure molecular epidemiologic questions.

Utility of the population genetic framework. For investigating many questions of interest to MRSA research, we favor the evolutionary genetic classification of clones provided by multilocus enzyme electrophoresis. One of the primary advantages of a genetically based framework for categorizing MRSA isolates is that for many bacterial species, virulence potential frequently is nonrandomly apportioned along

clonal lineages (40). In addition, population genetic analysis permits strong inferences regarding many aspects of the biology and virulence to be generated based on relationships among clonal lineages. For example, the recognition (27) that toxic shock syndrome (TSS) toxin 1-producing *S. aureus* strains recovered from ovine sources are genotypically distinct from the clone causing about 90% of all human TSS cases suggested that allelic differences exist in the TSS toxin 1 structural gene (*tst*) harbored by strains recovered from the different host species. This prediction was recently borne out by the identification of a second *tst* allele preferentially associated with ovine isolates, and, interestingly, the proteins encoded by the variant alleles differ in functional activity (22). In the present study, we identified *mec* in association with isolates of the major clone (here designated ET 15) responsible for more than 90% of TSS episodes and virtually all TSS cases with a urogenital focus (27), and it is noteworthy that some of the ET 15 isolates were recovered from patients with this disease (20). Our discovery that the *mec* gene is commonly harbored by five lineages (ET 2, ET 7, ET 9, ET 14, and ET 15) that are divergent from one another in overall chromosomal character and represent a large proportion of the breadth of multilocus enzyme diversity present in the species provides a population genetic context for future investigations of basic biological differences among MRSA clones.

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