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A single clone of *Staphylococcus aureus* causes the majority of cases of toxic shock syndrome

(genetic polymorphism/multilocus enzyme genotypes/clones/toxin)

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ABSTRACT **Genetic relationships among 315 isolates of the bacterium *Staphylococcus aureus* expressing toxic shock syndrome toxin-1 (TSST-1) recovered primarily from humans with toxic shock syndrome (TSS) in five countries on two continents were determined by analyzing electrophoretically demonstrable allelic variation at 20 chromosomal enzyme loci. Forty-nine distinctive electrophoretic types (ETs), representing multilocus enzyme genotypes, were identified. Cluster analysis of the ETs revealed two major phylogenetic divisions separated at a genetic distance of 0.35 and seven branches diverging from one another at distances ≥0.20. A single clone (ET 41) accounted for 88% of cases of TSS with a female urogenital focus and 53% of TSS cases involving nonurogenital (predominantly wound) infections. With few exceptions, strains representing different phylogenetic lines had characteristic TSST-1 gene (*tst*) restriction fragment length polymorphism patterns obtained by digestion of genomic DNA with *Cla* I. Strains recovered from ovine and bovine hosts with mastitis were genotypically distinct from the major human TSS clone. The expression of TSST-1 in cell lineages representing the total breadth of multilocus genotypic diversity in the species *S. aureus* as a whole is interpreted as evidence that the TSST-1 gene is evolutionarily old. The recovery of a single clone from the majority of individuals afflicted with TSS having a urogenital focus and from the genital tract of a large proportion of asymptomatic female carriers strongly suggests that this clone is especially well adapted for colonization of these anatomic sites.**

Toxic shock syndrome (TSS) was first described in 1978 (1) as a severe acute illness (characterized by high fever, erythematous rash, hypotension or shock, multiorgan involvement, and desquamation of the skin) of young children associated with infection with the bacterium *Staphylococcus aureus*. Two years later, it was recognized that TSS is a geographically widespread disease affecting mainly young, healthy, menstruating women, especially those using tampons (2). Most vaginal isolates of *S. aureus* from patients with TSS produce a chromosomally encoded toxin, designated as toxic shock syndrome toxin-1 (TSST-1) (3–5).

Evidence implicating TSST-1 as a major virulence factor in the pathogenesis of TSS has accumulated (6). Almost all strains recovered from patients with menstrual TSS, which account for about 90% of cases of TSS, synthesize TSST-1, whereas only 50–60% of isolates from nonmenstrual cases of TSS and 5–25% of strains causing other diseases produce this protein (3, 4, 7–10). When injected into animal hosts, purified TSST-1 produces many signs and symptoms characteristic of human TSS (11–14).

Numerous investigations have attempted to identify phenotypic characters marking TSS-associated strains. As a group, these strains more frequently exhibit proteolytic activity *in vitro* (15, 16), produce less hemolysis on sheep-blood agar (15–18), and less frequently harbor plasmids than do control strains (16, 19). TSS strains also differ from other *S. aureus* isolates in having a higher frequency of resistance to heavy metals and antibiotics (15, 20) and in other phenotypic characters (21–23).

It has been recognized for a number of years that many cases of menstrual TSS are caused by *S. aureus* strains of bacteriophage lytic group I (20, 24–26), but 30–40% of isolates are nontypeable with the panel of phages in the International Basic Set for phage typing *S. aureus* (25). Strain discrimination recently has been achieved by analysis of restriction fragment length polymorphism (RFLP) patterns of genomic DNA, with a segment of the TSST-1 structural gene (*tst*) used as a probe (19, 27, 28). However, neither phage subtyping nor RFLP analysis, as currently conducted, provides a basis for understanding the genetic structure of populations or associations of chromosomal background and virulence factor expression.

Several questions of importance for both medical bacteriology and evolutionary genetics remain unanswered. For example, data from RFLP analysis of the *tst* gene have been interpreted as evidence that strains recovered from cases of TSS represent more than one clonal lineage (27, 28), but the actual number of phylogenetic lines of *S. aureus* expressing TSST-1 is unknown. And controversy exists as to whether the TSST-1 structural gene is horizontally transferable in natural populations (29, 30). Because it has recently been recognized that many strains of *S. aureus* cultured from animal hosts synthesize TSST-1 (31), it is important to assess the amount of cross-infection occurring between host populations. Finally, there is no adequate explanation for the dramatic increase in the frequency of TSS cases recorded in North America and elsewhere in the late 1970s and early 1980s.

We have used multilocus enzyme electrophoresis, which yields estimates of diversity in the chromosomal genome and

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Abbreviations: ET, electrophoretic type; TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin-1; RFLP, restriction fragment length polymorphism.

the genetic relatedness of strains (32), to determine the genetic structure of natural populations of TSST-1-producing *S. aureus* causing menstrual TSS and other diseases. The objective of our research was to construct a molecular population genetic framework within which problems relating to toxin distribution, host and disease specificity, frequency of horizontal transfer of virulence genes, and the evolutionary origins of the organisms causing human and animal diseases can be studied.

We here report the results of an analysis of electrophoretically demonstrable allelic variation in 20 enzyme-encoding chromosomal genes in 315 isolates of *S. aureus* expressing TSST-1. Our study revealed that TSST-1 expression occurs in association with chromosomal backgrounds representing the full breadth of genotypic diversity in the species as a whole. We also have discovered that the organisms responsible for most cases of TSS with a female urogenital focus are members of a single distinctive clone. Our results suggest that the gene encoding TSST-1 is evolutionarily old and that the common TSS clone has special properties conferring strong affinity for human cervicovaginal surfaces.

MATERIALS AND METHODS

Bacterial Isolates. In total, 315-TSST-1-producing strains of *S. aureus* were analyzed. The sample included 293 human isolates recovered from the following sources: 187 isolates from epidemiologically unassociated cases of TSS with a urogenital focus (introitus, vagina, cervix), 47 isolates from individuals with TSS having a nonurogenital focus (e.g., wound abscesses, pulmonary infections), 28 isolates from cases of infection not involving TSS, and 25 carrier isolates from female urogenital sources. For each of six patients with TSS, we analyzed two cultures from different anatomic sites. Isolates from patients with TSS were from 14 states in the continental United States (n = 102), seven provinces in Canada (n = 48), Denmark (n = 14), Sweden (n = 21), and New Zealand (n = 2). Fourteen of the isolates had been recovered from human sources in 9 states between 1960 and 1976.

Of 22 animal isolates examined, 17 were recovered from cases of ovine mastitis in the German Democratic Republic (n = 9), France (n = 7), and Bulgaria (n = 1); and 5 isolates were from cases of bovine mastitis in the United States.

The actual frequencies of recovery of TSST-1-producing strains from urogenital and nonurogenital TSS cases is not accurately reflected by the numbers of isolates studied. Nonurogenital TSST-1-producing isolates, which account for only about 5–10% of all TSS cases, were overrepresented in our sample (21% of isolates from individuals with TSS), strictly for purposes of genetic comparison.

Electrophoresis of Enzymes. Isolates were grown at 37°C overnight in 150 ml of tryptic soy broth (Difco) on an orbital shaker (250 rpm) and harvested by centrifugation at 6000 × g for 10 min at 4°C. After suspension in 2 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 37–40°C for 30 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 ice-water cooling, and were centrifuged at 20,000 × g for 20 min at 4°C. The clear supernatant (lysate) was stored at −70°C.

Lysates were electrophoresed on starch gels and selectively stained for 20 metabolic enzymes by methods described by Selander *et al.* (32). The enzymes studied were aconitase, phenylalanine peptidase, phosphoglucose isomerase, carbamylate kinase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, mannitol-1-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutamate dehydrogenase, nucleoside phosphorylase, catalase, esterase, lactate dehydrogenase 1, lactate dehydrogenase 2, lactate dehydrogenase 3, alcohol dehydrogenase, maltose dehydrogenase, leucylglycylglycine peptidase, 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 almost all isolates, including many that lacked detectable plasmids, showed activity for all 20 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 20 enzyme loci, and distinctive combinations of electromorphs, corresponding to unique multilocus genotypes, were designated as electrophoretic types (ETs) (32).

Statistical Analysis. Single-locus genetic diversity among ETs was calculated from allele frequencies as $h = (1 - \Sigma x_i^2)\,[n/(n-1)]$, where x_i is the frequency of the ith allele and n is the number of ETs; mean diversity per locus (H) is the arithmetic average of h values over all loci. Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which different alleles were represented (mismatches) (32).

Toxin Assays. TSST-1 production by most strains was evaluated by double immunodiffusion after toxin was precipitated from culture fluid with ethanol and redissolved to 1/50th or 1/100th of the volume of the original culture medium. Toxin expression by some strains was assessed with a colony immunoblot assay (33).

RESULTS

Genetic and Genotypic Diversity. Sixteen of the 20 enzyme loci were polymorphic, with an average of 3.2 alleles per locus. Forty-nine distinctive multilocus genotypes (ETs) were identified, among which mean genetic diversity per locus (H) was 0.267 (Table 1). Of these 49 ETs, 35 were represented by single isolates and 14 by multiple isolates (range, 2–212 isolates).

Genetic Relationships Among Multilocus Genotypes. Estimates of the genetic relationships of the 49 ETs, based on allelic variation at the 20 enzyme loci, are summarized in the dendrogram in Fig. 1. The smallest observed genetic distance (0.05) between ETs corresponds to a single-locus difference, and the largest distance (0.35) corresponds to differences at 7 of the 20 loci assayed.

At a genetic distance of 0.20, there are seven branches, designated A through G. Clusters B, C, D, and F each consisted of seven or more ETs; cluster A was composed of two ETs, and the E and G lineages were each represented by a single ET. Clusters A–D and lineage E were separated from cluster F and lineage G at a distance of 0.35; these two

Table 1. Composition of a sample of 309 isolates of *S. aureus* producing TSST-1

Source	Isolates, no.	ETs, no.	H
Human urogenital TSS	187	24	0.246
Human nonurogenital TSS	47	10	0.279
Human non-TSS and carriers	53	13	0.262
Animal mastitis	22	8	0.171
Total	309	49	0.267
Entire species	2077	252	0.289

H is mean genetic diversity per locus among ETs. Entire species refers to 252 ETs represented by 2077 isolates (J.M.M., unpublished data).

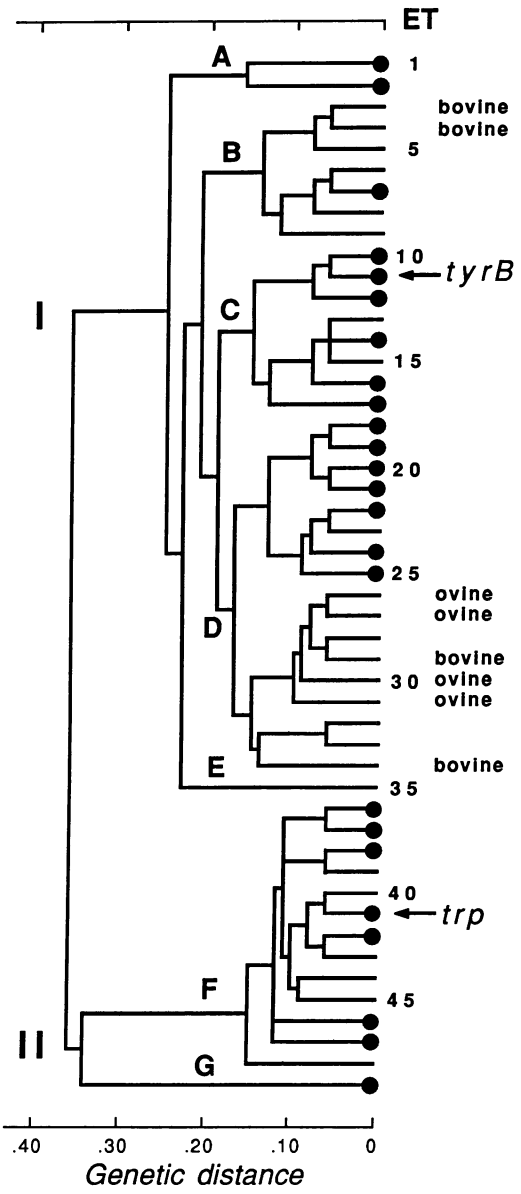

FIG. 1. Dendrogram showing genetic relationships of 49 ETs of 315 isolates of *S. aureus* producing TSST-1. There are two primary divisions (I and II) and seven major lineages (A–G). Closed circles denote ETs with one or more isolates recovered from patients with urogenital TSS. ETs with isolates from nonhuman (ovine and bovine) hosts are indicated. ETs 2, 3, 4, 14, 26, 27, 29, 30, 31, 34, and 41 were represented by isolates that produce enterotoxin C_1 in addition to TSST-1. The *tst* gene has been mapped near the *tyrB* locus in isolates of ET 11 and near the *trp* locus in isolates of ET 41 (27).

divisions are designated as I and II in Fig. 1. The 13 ETs in cluster F were represented by 78% of all isolates.

Distribution of ETs Among Host Species. There was no sharing of ETs among human, bovine, and ovine isolates. Bovine isolates were confined to ETs 3 and 4 (in cluster B) and ETs 29 and 34 (in cluster D), and ovine isolates represented ETs 26, 27, 30, and 31—all in cluster D.

Genetic Variation in Relation to Disease Episode. Within three groups of ETs represented by isolates recovered from (*i*) individuals with TSS having a urogenital focus, (*ii*) individuals with TSS not having a urogenital focus, and (*iii*) healthy female carriers and individuals with non-TSS infections, estimates of genetic diversity per locus among ETs (0.246, 0.279, and 0.262, respectively) were roughly equivalent to that in the total sample of ETs (0.267) (Table 1), reflecting the fact that isolates of each group are of a wide variety of chromosomal enzyme genotypes. But isolates from animals with mastitis showed a reduced level of variation among ETs (0.171).

Only four (29%) of the 14 ETs with multiple isolates were represented by isolates cultured from more than one source group. Among the 187 isolates recovered from urogenital TSS cases, 164 (88%) were ET 41. Moreover, 25 (53%) of the 47 isolates recovered from patients with TSS having a nonurogenital focus, including six individuals with TSS and influenza (the Thucydides syndrome; refs. 34 and 35), also were ET 41. No isolates of ET 41 were cultured from animal sources.

Paired isolates recovered from different anatomic locations of the same patient were identical in multilocus enzyme genotype.

Geographic Distribution of ETs. Individual ETs may have wide, even intercontinental, distributions. For example, isolates of ET 41 in our sample were recovered from humans in 14 states of the continental United States, seven provinces in Canada, Denmark, Sweden, and New Zealand. Seventy-eight percent of all isolates from TSS patients in the United States were ET 41, which was also commonly represented among isolates from cases of TSS in Canada (94%), Denmark (100%), Sweden (86%), and New Zealand (50%).

Multilocus Enzyme Genotypes and *tst* RFLP Patterns. Seven RFLP patterns consisting of one (six patterns) or two (one pattern) fragments of various sizes have been demonstrated by probing genomic DNA with a 297-base-pair *Hin*dII–*Bam*HI segment of the *tst* gene (28). ETs 3, 4, 11, 14, 27, 30, and 41 each had a distinctive *tst* RFLP pattern (28); with few exceptions, isolates having the same *tst* RFLP pattern were of identical multilocus enzyme genotype (data not shown). We noted one instance in which two isolates with the same RFLP pattern were of different ETs (differing at 2 of the 20 enzyme loci assayed), and, conversely, we identified two cases in which two isolates of the same ET had different patterns. One of the isolates in the latter category was strain RN 4993, which is unusual in possessing two segments of DNA that hybridize with the *tst*-specific probe (28). Most isolates of ET 41 were of a single *tst* RFLP pattern, which has been described elsewhere (27, 28).

DISCUSSION

Primary Discovery. Using allelic enzyme variants detected by electrophoresis to index levels of genetic diversity and to estimate genetic relationships among strains of *S. aureus*, we have found that the majority (88%) of TSST-1-producing isolates recovered from patients with TSS having a urogenital focus, including individuals in North America, northwestern Europe, and New Zealand, are identical in multilocus enzyme genotype. Because evolutionary convergence to the same multilocus enzyme genotype is highly unlikely (36), the simplest explanation for the repeated recovery of TSST-1-positive isolates of the same ET is that they share recent lineal descent from a common precursor cell: the isolates represent a clone. This interpretation is supported by the observation that isolates of ET 41 also share many phenotypic traits (37, 38).

Our results also indicate that isolates of the common TSS-causing cell line (lineage F, Fig. 1) are somewhat distantly related to other TSST-1-producing strains of *S. aureus*. Isolates of ETs in lineages F and G (division II) are differentiated from isolates in division I at a genetic distance ≈ 0.35, which reflects allelic differences, on average, at seven of the chromosomal loci compared. Given this degree of overall genetic differentiation, it would not be surprising to find that isolates of ETs in lineage F regulate expression of the *tst* gene (or other genes whose products mediate virulence) differently than do isolates in the other lineages. We

note that a genetic distance of 0.35 exceeds the level of divergence at which distinct evolutionary lineages of *Bordetella* spp. specialize on different mammalian host species (39, 40).

The widespread recovery of isolates of a single clone from most cases of TSS with a urogenital focus implies the existence of an effective mechanism for rapid intercontinental dispersal. The failure to recover isolates of ET 41 from nonhuman hosts (Fig. 1 and J.M.M., unpublished data) effectively eliminates the likelihood that animals are important in the transmission of this clone. Because TSST-1-producing isolates, including those of ETs in lineage F, can be recovered from a variety of human body surface sites and from diseases other than TSS (Table 1; J.M.M., unpublished data), it is most likely that transmission usually occurs by human–human contact. Inasmuch as contamination of catemenial products does not appear to be a mode of spread, introduction of isolates into the vaginal vault probably occurs by autoinoculation.

Inferences from Evolutionary Genetic Data. Can the population genetic framework generated by multilocus enzyme electrophoresis help to explain the marked increase in frequency of TSS episodes that occurred in geographically widespread areas in the late 1970s and early 1980s? Our results show that the *tst* gene occurs in a large number of clonal lineages representing the total breadth of genetic diversity in the species *S. aureus* as a whole. Analysis of 2077 isolates from many disease sources detected a total of 252 ETs, but genetic diversity was not increased significantly (Table 1), and cluster analysis of these ETs did not reveal additional major lineages. This observation and the fact that isolates of ET 41 represented 24% of the sample of TSST-1-producing strains recovered before 1978 strongly suggest that the *tst* gene did not recently evolve in or was not recently acquired by *S. aureus*.

One hypothesis accounting for the temporal increase in the frequency of TSS disease postulates an episode of periodic selection (41, 42) involving a fitness mutation occurring in a cell representing ET 41. However, because TSS increased in frequency in many different regions in the same short period, we do not favor this hypothesis. Alternatively, the increase in number of TSS cases may have been occasioned not by a genetic change in the bacterium itself but by modifications in the host population so that the fitness of organisms of the already existing ET 41 TSST-1-positive genotype increased relative to that of other cell lines. According to this hypothesis, any alteration of the ecological niche occupied by toxin-positive cells already widely dispersed in human populations that results in a growth advantage, increased toxin production, or both, could produce a "bloom" of TSS cases. Thus, a change in the character of catemenial products, perhaps associated with decreasing levels of anti-TSST-1 antibody in human populations, might cause a rapid increase in disease frequency.

The association of TSST-1 production with virtually the full breadth of phylogenetic diversity present in *S. aureus* (Fig. 1; J.M.M., unpublished data) suggests that this trait is a primitive condition in *S. aureus* that has been retained in the course of evolutionary events generating genomic diversity in natural populations. An alternative hypothesis to account for the phylogenetically widespread distribution of the *tst* gene postulates multiple episodes of horizontal transfer and recombination in many toxin-negative lineages. Evidence of horizontal transfer is provided by the occurrence of the *tst* gene in at least two different regions of the chromosome in strains representing two relatively divergent evolutionary lineages (Fig. 1). However, attempts to transfer the gene through bacteriophage lysogenic conversion and plasmid-mediated processes have not been successful (30).

If, as we are suggesting, the gene encoding TSST-1 is evolutionarily old, there should exist in natural populations allelic variant strains differing in nucleotide and, perhaps, amino acid sequence. Consistent with this notion is the recent identification of antigenic diversity among TSST-1 proteins (31).

Adapted Clone Hypothesis. It is important to explain why isolates of a clone marked by multilocus enzyme genotype ET 41 have been responsible for an unusually large proportion of TSS cases. There are at least two hypotheses that could account for the association of isolates of ET 41 with 88% of urogenital TSS cases. In the cervicovaginal milieu, isolates of the clone marked by ET 41 may regulate expression of the toxin gene (or other genes whose products are critical for virulence) differently, on average, than do isolates of other clones, perhaps as a consequence of allelic variation in the *tst* gene itself or in the accessory gene regulator *agr*, a locus coding for a trans-activator of many exoprotein genes whose products are putatively involved in virulence (43). An alternative but not mutually exclusive hypothesis is that isolates of ET 41 are more prone to colonize the human vagina and, hence, are widely dispersed in an ecological niche of great consequence in TSS. Under the latter "adapted clone" hypothesis, isolates of ET 41 are responsible for most vaginal cases of TSS because this clone has a special affinity for the cervicovaginal milieu, perhaps (but not necessarily) as a consequence of variation in regulation of toxin-gene expression. Therefore, the chance of colonization by an isolate of this clone is much greater than that for other clones. Consistent with this idea is the observation that 28% of isolates of *S. aureus* from the introitus, vagina, or cervix of epidemiologically unassociated healthy carriers or women with non-TSS urogenital symptoms were ET 41 or closely allied ETs; and no other single multilocus enzyme genotype accounted for more than 12% of normal vaginal isolates. Although many of these isolates produce TSST-1, some do not, probably because of an absence of the *tst* structural gene. With the recent realization that isolates of different phylogenetic lines of a number of bacterial pathogens are associated with distinctive clinical syndromes (39, 40, 44, 45), it is reasonable to suggest that adapted clones are a common theme in microbial pathogenicity.

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