Occurrence of the Enterotoxin Gene Cluster and the Toxic Shock Syndrome Toxin 1 Gene among Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus* Is Related to Clonal Type and *agr* Group

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Analysis of 177 methicillin-resistant *Staphylococcus aureus* (MRSA) strains for the presence of *egc* and *tst* revealed that 60 strains carried at least one of the tested genes. MRSA strains were classified by pulsed-field gel electrophoresis into four clones belonging to *agr* groups I and III. Toxin genotypes were related by clonal type and *agr* group.

The emergence and spread of methicillin-resistant *Staphylococcus aureus* (MRSA) has posed a clinical threat worldwide. Staphylococcal enterotoxins, enterotoxin-like superantigens, and the toxic shock syndrome toxin 1 that belong to pyrogenic toxin superantigens (PTSAgs) are considered major virulence factors (5, 11). The enterotoxin gene cluster (*egc*) includes five enterotoxin-like superantigen genes (*seo*, *sem*, *sei*, *sen*, and *seg*) and two pseudogenes (*ψent1* and *ψent2*) (6, 8, 11). Recently, a new toxin, SEU, encoded by the *egc* operon (*egc2*), was identified to result from sequence divergence in the region of the pseudogenes *ψent1/ψent2* (9).

Expression of virulent genes in *S. aureus* is controlled by the accessory gene regulator (*agr*), with a polymorphism in the sequence of the autoinducing peptide and its receptor, according to which clinical strains can be divided into four *agr* groups (I to IV) (7, 10). *S. aureus* strains causing specific syndromes were linked to certain *agr* types (7). Thus, the purpose of the present study was to investigate the presence of the *egc* operon and *tst* in a set of 177 MRSA isolates collected from different patients admitted to the University Hospital of Patras, Greece, during 2001 to 2003, to determine the clones of isolates, and to correlate them with the *agr* groups and toxin gene profile.

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All 177 MRSA isolates were tested by the disk diffusion method against various antistaphylococcal agents (12). Oxacillin MICs were determined by the agar dilution method (13), and the presence of the *mecA* gene was determined by PCR (14).

Clones were defined by pulsed-field gel electrophoresis of SmaI digests of chromosomal DNAs performed in a CHEFF DR III apparatus (Bio-Rad Laboratories, Richmond, California) according to published protocols (4, 15) and were

compared to previously identified clones existing in our hospital (1).

agr typing was carried out by PCR as described previously (10) with modified thermal conditions as follows: predenaturation step for 5 min at 94°C, 25 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, extension for 1 min at 72°C, and a final extension step for 7 min at 72°C (J. Etienne and M. Bes, personal communication). Fri913 (*agr* class I), Fri137 (*agr* class II), ATCC 49775 (*agr* class III), and A920211 (*agr* class IV) were used as reference strains.

The sequences corresponding to *seo*, *sem*, *sei*, *ψent1/ψent2* (*ent*), *sen*, and *seg* included in the *egc1* operon and *tst* were detected by PCRs (6, 7). Fri137 and Fri913, carrying the *egc2* operon and *tst*, respectively, were used as reference strains.

The presence of *seu* characterizing the *egc2* operon (*seo*, *sem*, *sei*, *seu*, *sen*, and *seg*) was investigated by HindIII restriction fragment length polymorphism of the amplified *ent* region. The *ent* region of the *seu*-negative strain A900322 harboring *egc1* (9) carries a unique restriction site for the HindIII enzyme at nucleotide 456, resulting in two restriction fragments of 456 and 181 bp. This region does not exist in the corresponding amplified part of the *seu*-positive strain Fri137 (GenBank accession no. AY205306) carrying *egc2* (9). A restriction reaction in a final volume of 20 µl, containing 15 units HindIII (Promega, Madison, WI), 1× reaction buffer (Promega), and 7 µl of PCR product, was performed at 37°C with overnight incubation. Lambda (λ) DNA was used as the restriction enzyme marker.

The 177 MRSA isolates with distinct antibiotic resistance phenotypes were classified into four clones, as previously described (1). All multiresistant isolates were characterized as clone B (48 strains or 27%). Most isolates (103 of 177 or 58%) belonged to clone C, expressing resistance to beta-lactams and fusidic acid (3). Three strains (2%) resistant to beta-lactams, erythromycin, and ciprofloxacin were characterized as clone F. Clone A included 23 isolates (13%) resistant only to beta-lactams.

Fifty-two isolates (29%) of clones B and F and a unique strain of clone C that did not possess the Panton-Valentine leukocidin (PVL) genes (3) belonged to *agr* class I. One hun-

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TABLE 1. Superantigen profiles and *agr* class characterization of methicillin-resistant *S. aureus* isolates in relation to clones and clinical samples

<i>agr</i> group (n [%])	Clinical sample source(s)	No. of positive PCR results (PFGE type) for:												Total (%)
		<i>tst/egc2</i>	<i>egc1</i>	<i>egc2</i>	<i>sei</i>	<i>sei/seg</i>	<i>ent-seu</i>	<i>sem</i>	<i>seg</i>	<i>tst/sei/seg/ent-seu/</i> <i>sen/seg</i>	<i>tst/seg</i>	<i>seo</i>	None	
I (52 [29])	Abscess						1 (B)			1 (B)			20 (B)	22 (12)
	Skin lesions												2 (B)	2 (1)
	Wounds		3 (F)		3 (B)		3 (B)			1 (B)			11 (B)	21 (12)
	Blood		1 (C)								1 (B)		1 (B)	3 (2)
	Urine				1 (B)								3 (B)	4 (2)
III (125 [71])	Abscess	3 (A)			5 (C)			1 (C)					19 (C)	28 (16)
	Skin lesions	2 (A)					3 (C)					1 (C)	23 (C)	29 (16)
	Wounds	15 (A)			4 (C)		3 (C)	2 (C)	3 (C)				36 (C)	63 (36)
	Blood	1 (A)		1 (A)									2 (C)	4 (2)
	Urine	1 (A)												1 (1)
Total (%)		22 (12)	4 (2)	1 (1)	13 (7)	6 (3)	4 (2)	3 (2)	3 (2)	2 (1)	1 (1)	1 (1)	117 (66)	177 (100)

dred twenty-five (125 or 71%) isolates of clones A and C, with the exception of the aforementioned PVL-negative strain, belonged to *agr* class III (Table 1).

Among these 177 strains, 117 (66%) did not carry any

PTSag tested. Sixty MRSA isolates positive for at least one of the tested genes were classified into 11 genotypes (Table 1). The *tst* gene was always detected in combination with *egc* genes.

Twenty-three *egc*-positive isolates and six clinical isolates carrying the *ent* region (ψ ent1/ ψ ent2-positive) were *seu* positive (*egc2*). The remaining four *egc*-positive isolates did not possess the *seu* gene (*egc1*) (Fig. 1). The Fri137 reference strain carried the *seu* gene, as expected (9).

The genotypes *tst/egc2* and *egc2* belonging to clone A were classified into *agr* group III. Four strains possessing *egc1*, classified to clones F (3 strains) and C (1 PVL-negative strain), belonged to *agr* group I (Table 1).

Figure 2 shows the distribution of specific genotypes for each year. Isolates harboring all tested genes reduced with time, whereas those which carried no genes remained high.

One-third (60/177) of the MRSA isolates tested carried a number of genes, ranging from one to seven, with an extensive variation between individual strains and a reducing tendency during the study period. Coexistence of *tst* with enterotoxin genes has been reported but not exclusively for all tested isolates (7), as shown in the present study. The prevalence of *egc2* in our study is much higher than first reported (9). Discrepancies in the frequency of PTSag genes from different countries are expected, since they are associated with mobile genetic elements and strains may gain a different number of genes.

The variable possession of *egc* genes suggests a more heterogeneous composition of this operon, leading to the emergence of the newly described *seu* gene (9) or the allelic variants of *seg*, *sei*, *sen*, and *seu* (2, 6). Identification of variable toxin gene profiles may be explained by horizontal transfer, since open reading frames for putative transposases are located nearby, although it is limited among lineages (7, 8).

Identification of *agr* groups classified the majority of our strains into group III and to a lesser extent into *agr* group I. In previous studies, most of the MRSA isolates were *agr* group I or II (7). Investigation of the relationship between *agr* groups, clones, and toxin gene distribution reveals that the presence of tested genes is clone related. Classification of *tst*-positive isolates in *agr* group III has been already reported among methicillin-susceptible *S. aureus* isolates (7).

The basic unit of bacterial pathogenicity may reside on the

FIG. 1. Analysis of *ent* region by PCR-restriction fragment length polymorphism with HindIII. Lanes 1 and 19, 100-bp ladder as molecular size marker. (A) Lane 2, λ DNA digested by HindIII; lane 3, reference strain Fri137, *seu* positive (*egc2*); lanes 4 to 7, strains of clone B with *ent-seu* genotype; lanes 8 and 9, strains of clone B with *tst/sei/seg/ent-seu/*
sen/seg genotype; lanes 10 to 18, strains of clone A with *tst/egc2* genotype. (B) Lanes 2 to 8, strains of clone A with *tst/egc2* genotype; lanes 9 to 11, strains of clone F with *egc1* genotype; lane 12, strain of clone A with *egc2* genotype; lanes 13 to 15, strains of clone A with *tst/egc2* genotype; lane 16, strain of clone C with *egc1* genotype; lanes 17 and 18, strains of clone A with *tst/egc2* genotype.

FIG. 2. Distribution of different toxin gene profiles among clinical MRSA isolates during the 3-year study period.

clone or lineage, since it expands, possessing particular combinations of virulence and regulatory genes in the appropriate genetic background (7). The sensitive clone A possessing all tested genes does not seem to be a threat to our patients, since it decreases with time.

In conclusion, one-third of 177 MRSA clinical isolates carried genes of the PTSAg family, with a reducing tendency during the study period. *tst* always coexisted in the same isolate with other genes of the *egc* operon. Clone A, which harbors all or the majority of tested toxin genes (*tst/egc2* or *egc2*), expresses a sensitive phenotype; however, it could not correlate with a specific clinical syndrome. This is the first report of a representative collection of MRSA in Greece. A clonal relationship with *agr* group classification and toxin gene profile was identified, and it remains to be seen whether a specific lineage spread may occur. Identification of strains with variable toxin gene profiles belonging to different clones indicates that *egc* genes are not strictly linked and a multitude of combinations among themselves and other PTSAGs (*tst*) may occur in bacterial populations.

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REFERENCES

- Aires de Sousa, M., C. Bartzavali, I. Spiliopoulou, I. Santos Sanches, M. I. Crisóstomo, and H. de Lencastre. 2003. Two international methicillin-resistant *Staphylococcus aureus* clones endemic in a university hospital in Patras, Greece. *J. Clin. Microbiol.* **41**:2027–2032.
- Becker, K., A. W. Friedrich, G. Peters, and C. von Eiff. 2004. Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SEIM, SEIO and SEIN. *Mol. Nutr. Food Res.* **48**:488–495.
- Chini, V., E. Petinaki, A. Foka, S. Paratiras, G. Dimitracopoulos, and I. Spiliopoulou. 2006. Spread of *Staphylococcus aureus* clinical isolates carrying Pantón-Valentine leukocidin genes during a 3-year period in Greece. *Clin. Microbiol. Infect.* **12**:29–34.
- de Lencastre, H., I. Couto, I. Santos, J. Melo-Cristino, A. Torres-Pereira, and A. Tomasz. 1994. Methicillin-resistant *Staphylococcus aureus* disease in a Portuguese hospital: characterization of clonal types by a combination of DNA typing methods. *Eur. J. Clin. Microbiol. Infect. Dis.* **13**:64–73.
- Dinges, M. M., P. M. Orwin, and P. M. Schlievert. 2000. Exotoxins of *Staphylococcus aureus*. *Clin. Microbiol. Rev.* **13**:16–34.
- Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougél, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina. 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J. Immunol.* **166**:669–677.
- Jarraud, S., C. Mougél, J. Thioulouse, G. Lina, H. Meugnier, F. Forey, X. Nesme, J. Etienne, and F. Vandenesch. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. *Infect. Immun.* **70**:631–641.
- Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu. 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* **357**:1225–1240.
- Letertre, C., S. Perelle, F. Dilasser, and P. Fach. 2003. Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. *J. Appl. Microbiol.* **95**:38–43.
- Lina, G., F. Boutite, A. Tristan, M. Bes, J. Etienne, and F. Vandenesch. 2003. Bacterial competition for human nasal cavity colonization: role of staphylococcal *agr* alleles. *Appl. Environ. Microbiol.* **69**:18–23.
- Lina, G., G. A. Bohach, S. P. Nair, K. Hiramatsu, E. Jouvin-Marche, and R. Mariuzza, for the International Nomenclature Committee for Staphylococcal Superantigens. 2004. Standard nomenclature for the superantigens expressed by *Staphylococcus*. *J. Infect. Dis.* **189**:2334–2336.
- National Committee for Clinical Laboratory Standards. 2003. Performance standards for antimicrobial disk susceptibility tests, 8th ed. Approved standard M2–A8. National Committee for Clinical Laboratory Standards, Wayne, Pa.
- National Committee for Clinical Laboratory Standards. 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard M7–A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
- Petinaki, E., A. Arvaniti, G. Dimitracopoulos, and I. Spiliopoulou. 2001. Detection of *mecA*, *mecR1* and *mecI* genes among clinical isolates of methicillin-resistant staphylococci by combined polymerase chain reactions. *J. Antimicrob. Chemother.* **47**:297–304.
- Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**:2233–2239.