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# Clonal study of enterotoxin-B producing strains of *Staphylococcus aureus*

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## SUMMARY

Sixty-nine *Staphylococcus aureus* strains, 39 of which produced staphylococcal enterotoxin B (SEB+) and 14 of which were associated with toxic shock (TS+), were studied using the following markers: serotyping, phage typing, antibiotyping, ribotyping, zymotyping and pulsed-field electrophoresis typing. Analysis of the results showed that the enterotoxin B producing strains were derived from at least three clones: the first two consisted of methicillin-susceptible strains, while the third included the methicillin-resistant (MRSA) strains. TS+ strains of non-genital origin appeared to be distributed between the three clones, with no specific characters.

## INTRODUCTION

The toxic shock syndrome toxin-1 (TSST-1) of *Staphylococcus aureus* is responsible for staphylococcal toxic shock (TS). Most of the strains of genital origin and 50% of others belong to the same clone [1]. Among the other *S. aureus* toxins, staphylococcal enterotoxin B (SEB) has been associated with disease recognized post-mortem [2, 3]. Responsible mainly for food poisoning, it has also been associated with toxic shock [4–6]. The properties of SEB-producing strains (SEB+) are well documented; Asheshov and co-workers [7] demonstrated that the strains in the 94/96 phage complex were very often SEB+; Melconian and colleagues [8] observed that these strains were lysed mainly by phages of groups II and V: and Dornbusch and Hallander [9] have demonstrated a relationship between oxacillin resistance and toxin production. Recently, Lee and colleagues [10] have shown that TS-associated strains produced either TSST-1, enterotoxin B or, exceptionally, both; they have also shown that SEB+ strains belonged to the same zymotype, suggesting a clonal origin. The aim of the present study was to confirm or refute the existence of an enterotoxin-B producing cell clone by studying the phenotypic and genotypic characters of SEB+ strains (whether or not associated with TS) as well as non-producing strains, using six different methods: phage typing, serotyping, antibiotyping, zymotyping, ribotyping and pulsed-field electrophoresis typing.

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## MATERIALS AND METHODS

### *Strains*

Thirty-nine SEB+ strains (14 of which were associated with TS) and 30 non-enterotoxin B-producing strains (SEB negative = SEB−) were selected by the Centre National de Référence des Staphylocoques (Lyon, France). They were not epidemiologically related and were isolated between 1980 and 1989 from different patients and hospital departments. None had been included in the study carried out by Melconian and colleagues [8]. None produced both enterotoxin B and another enterotoxin or TSST-1 (apart from Sample No. 5332 that also produced enterotoxin D: SED+). The negative control strains produced no toxin. Production of enterotoxin B and other toxins was demonstrated by an ELISA method [11].

### *Phage typing*

The 23 bacteriophages of the International basic set of *S. aureus* were used according to the recommendations of the International Committee on Systematic Bacteriology, Subcommittee on Phage Typing of Staphylococci [12]. The strains lysed by phages 29, 52, 52A, 79 and 80 belonged to phage group I: those lysed by phages 3A, 3C, 55 and 71 belonged to group II; those lysed by phages 6, 42E, 47, 53, 54, 75, 77, 83A, 84 and 85 belonged to group III; those lysed by phages 94 and 96 belonged to group V; those lysed by phages 81 and 95 belonged to group D: and those lysed by miscellaneous phage groups belonged to mixed group M.

### *Serotyping*

Serotyping was performed by agglutination, according to a previously described method [13, 14].

### *Antibiotic susceptibility pattern*

The minimum inhibitory concentrations (MIC) were determined by agar dilution method [15]. The antibiotics used were: penicillin G, oxacillin, streptomycin, kanamycin, neomycin, gentamicin, tobramycin, erythromycin, spiramycin, lincomycin, pristinamycin, tetracycline, minocycline, chloramphenicol, pefloxacin, fusidic acid, vancomycin, teicoplanin, fosfomycin, rifampin, and trimethoprim-sulfamethoxazole. Oxacillin MIC was determined in Mueller Hinton agar containing 4% NaCl. Beta-lactamase was determined using chromogenic cephalosporin (Cephinase, bio-Mérieux, Lyon, France). The resistance profile obtained was called antibiotype.

### *Multilocus enzyme electrophoresis = zymotyping*

Strains were grown on solid medium (Gelose Nutrient Agar Code CM3, Oxoid, Basingstoke, Hants) and suspended in 7 ml buffer (0·075 M Tris, 0·06 M glycine) (pH 8·7). After washing three times, the enzyme extracts were resuspended in 0·71 mg/g lysostaphin-containing Tris–glycine buffer (Sigma). After 30 min contact at 25 °C, the extracts were centrifuged, the supernatants were collected, filtered (0·22 $\mu$ millipore), and aliquots were kept at −20 °C. Demonstration of enzyme activity was carried out on starch agar (11·5%) (Connaught Labs, Canada), according to a previously-described method [14]. Twenty-one enzymes were detected: glucose-6-phosphate dehydrogenase (G6PD), peptidase (PEP),

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aconitase (ACO), adenylate kinase (ADK), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), alanine dehydrogenase (ALD), phospho-gluco-mutase (PGM), phospho-gluco-isomerase (PGI), lactate dehydrogenase (LDH), esterases 2, 3, 5, 6 (ES2, 3, 5, 6), indophenol oxydase (IPO), hydroxybutyrate dehydrogenase (HBD), threonine dehydrogenase (THD), lysine dehydrogenase (LYD), leucine dehydrogenase (LED), and alcohol dehydrogenase (ADH). At least two electrophoresis migrations were carried out for each sample. Electrotypes were determined as previously described [16].

*Ribotyping*

DNA was extracted following a published micromethod [17]. Total DNA (approximately 100 $\mu$g) was purified by floating filter dialysis (Millipore, VS 0·025 $\mu$). Total DNA (3–5 $\mu$g) was cleaved with restriction endonuclease *Eco*R I (Boehringer, Mannheim), 1 U/$\mu$g DNA, according to the manufacturer's instructions. Each gel digest was repeated at least twice to ensure that gels represented complete and not partial digest. Restriction DNA fragments were electrophoretically separated on horizontal gels of 0·8% (w/v) agarose in TBE buffer (0·089 M Tris–borate, 0·089 M boric acid and 0·002 M EDTA) at 2·5 V/cm for 18 h at room temperature. The gels were stained with ethidium bromide (1 $\mu$g/ml) and photographed under UV light using a Wratten 2A filter with a polaroid type 55/pn film. After electrophoresis, agarose gels were transferred for 1 h to a Hybond N membrane (pore size 0·45 $\mu$m, Amersham) using the Vacugene system (Pharmacia, France). The membranes were washed in 2 × SSC buffer (1 × SSC is 0·15 M NaCl plus 0·015 M trisodium citrate), dried 20 min at 37 °C, then exposed for 4 min under UV light to cross-link DNA to nylon. The blots were stored at room temperature until use. The membranes were treated with the reagents contained in an acetylaminofluorene kit (AAF Kit, Eurogentec, Liège, Belgium): thus they were prehybridized, hybridized with 16S-23S rRNA from *Escherichia coli* previously labelled with AAF (AAF rRNA) and revealed by an immunoenzymatic reaction following the manufacturer's instructions. The DNA fragment size marker Raoul I, also enclosed in the AAF kit, was hybridized with AAF-pBR322. The hybridized fragments appeared within 5 min to 1 h with a purple colour. The sizes of the rRNA gene restriction fragments were calculated according to a previously described method [18].

*Macrorestriction analysis of genomic DNA using pulsed-field electrophoresis*

Chromosomal DNA was isolated according to a previously described method [19] modified by Lina and co-workers [20]. The strains were grown in Brain-Heart broth for 3–4 h in a water-bath. After centrifugation and washing of bacteria, the suspension was adjusted to 1·8 absorbance units in TNEE buffer (10 mM Tris–HCl, 1 M NaCl, 10 mM EDTA and 10 mM ethylene glycol (aminoethyl ether) tetraacetic acid: EGTA). The agarose plugs were obtained by mixing 1 vol of calibrated suspension with 1 vol 2% Incert agarose (FMC Bioproduct). The solidified plugs were placed in 1 ml of lysis buffer: 6 mM tris-base, 100 mM EDTA, 1 M NaCl, 0·5% Brij 58 (Polyoxyethylene 20 cetyl ether, Sigma), 0·2% sodium deoxycholate, 0·5% saccharose (pH 7·6), 50 mg/ml lysostaphin (Applied Microbiology Inc.) and 500 mg lysozyme (Sigma); they were then incubated for 24 h at 37 °C. The lysis



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buffer was substituted for proteolysis buffer (0·25 mM EDTA, 20 mM EGTA, 1% sarcosyl, pH 9·0, 250 mg/l proteinase K). After incubation for 24 h at 60 °C, the plugs were washed three times with 5 ml TE buffer (pH 8·0) containing 1 ml phenyl-methyl-sulfonyl-fluoride at 4 °C and three times with TE buffer (pH 8·0). Digestion of plugs was then performed for 18 h at 25 °C in 200 $\mu$l of restriction buffer containing 30 U of *Sma*I (Boehringer, Mannheim). Digestion fragments were separated in 0·8% agarose gel by pulsed-field electrophoresis (CHEF System of Bio-Rad) for 22 h at 12 °C (pulse time: 25 s). The gels were then stained with ethidium bromide and photographed. Each strain was tested three times.

*Statistical analysis: genetic relationships, dendrograms*

For zymograms, the genetic diversity at an enzyme locus was calculated from the allele frequencies as $h = 1 - \Sigma x_i(n/n-1)$ where $x_i$ is the frequency at the $i^{th}$ allele and $n$ is the number of ETs. Mean genetic diversity per locus is the arithmetic mean of $h$ values for all loci [16]. For zymotypes, ribotypes and pulsed-field electrophoretypes, similarity of strains was calculated with the DICE coefficient, and hierarchical clustering was done by the unweighted pair group method with arithmetic average (UPGMA) [21]. Statistical significance was calculated according to the Chi-squared method.

## RESULTS

*Phage typing, serotyping*

The 39 SEB+ strains belonged to the following phage groups: group II, 11 strains; group III, 4 strains; group V, 17 strains; mixed groups M, 4 strains: 3 strains were not typable. Moreover, the serotype of group-II strains was h2/263-2/l, and that of group-V strains was b1/c1/o/p.

The 30 strains that failed to synthesize enterotoxin B belonged to the following phage groups: group I: 1; group II, 6; group III, 1; group V, 1; group D, 1: mixed groups, 12; 8 strains were not typable.

*Antibiotyping*

Fifteen of the 39 SEB+ strains (38%) were susceptible to penicillin G, and 30 (77%) to oxacillin. The oxacillin MIC of five of the strains was greater than 16 mg/l; these same strains were also multiresistant (aminoglycosides, macrolides, tetracyline, fluoroquinolone), whereas the MICs of four others were equal to 4 mg/l, with no multiresistance (borderline strains) [22].

Resistance to penicillin G of non-toxin-producing strains was demonstrated by 16 out of 30 (53%) of the strains; all susceptible to oxacillin.

*Zymotyping*

Ten of the 21 enzymes tested were monomorphic (1 allele) and 11 were polymorphic (2–27 alleles). The mean number of alleles per locus was 5·6. The total number of electrophoretypes (ET) was 52. The smallest genetic distance observed was 0·04; it was in agreement with a one-locus difference, while the greatest distance was 0·32 for a 5-locus difference. The mean genetic diversity was 0·265 for all the strains, 0·154 for TS+, 0·220 for SEB+ and 0·295 for SEB− strains. Analysis of the dendrogram (Fig. 1) revealed that SEB+ strains could be divided

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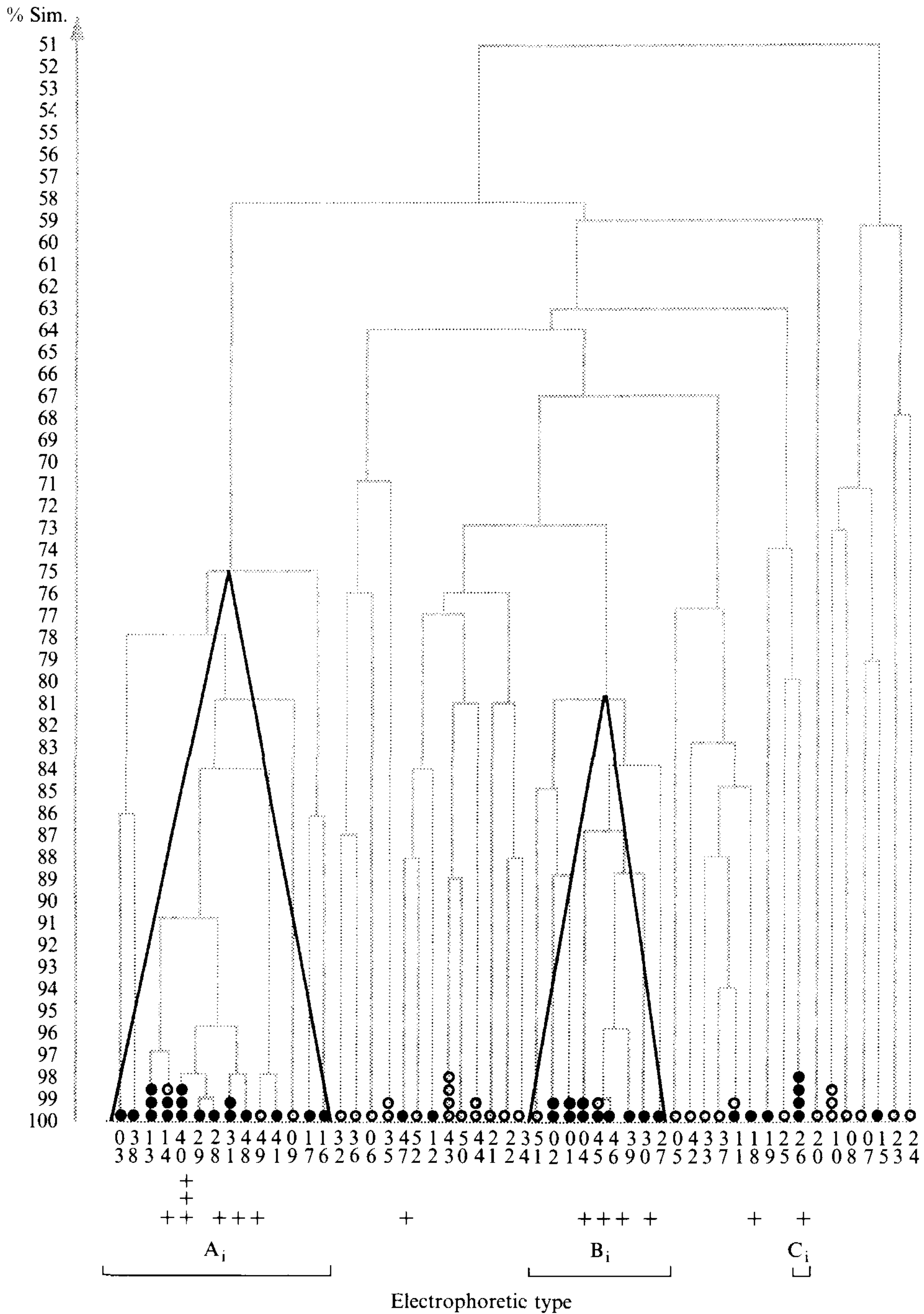


Fig. 1. Dendrogram of the percent similarity between electrophoretic types of *S. aureus* SEB+ (●) and *S. aureus* SEB− (○) strains. Toxic shock syndrome associated strains (TS) are represented +.



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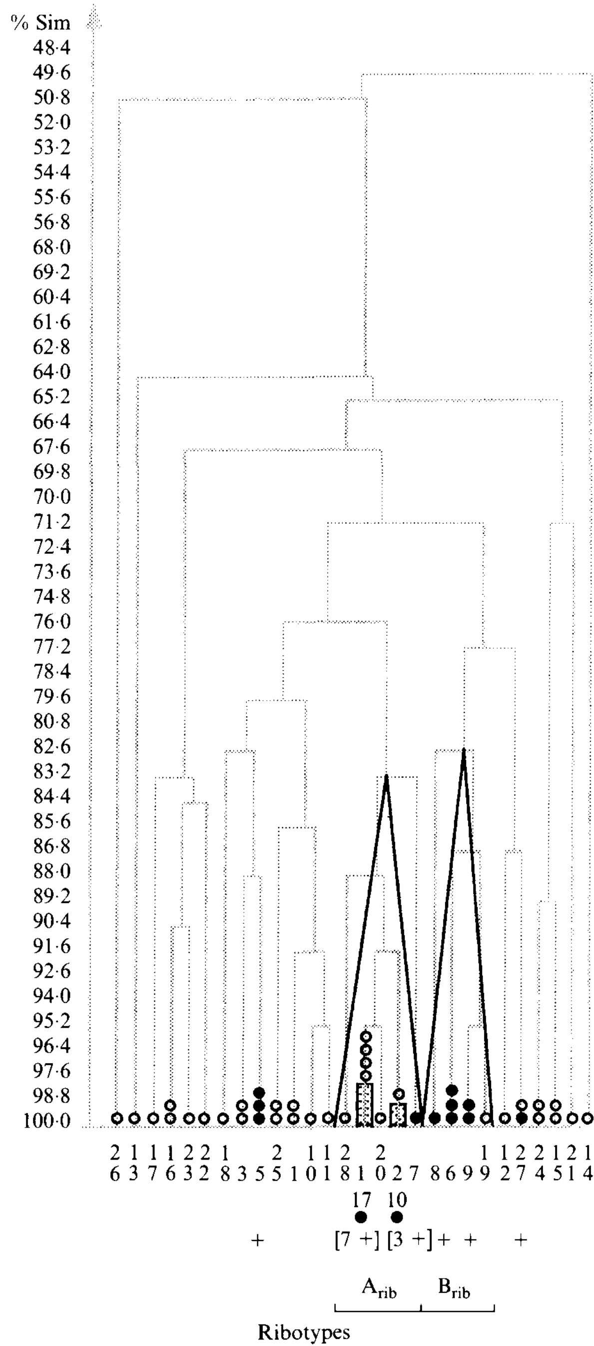


Fig. 2. Similarity dendrogram of *S. aureus* SEB+ (●) and *S. aureus* SEB− (○) strains. TS strains are noted +.

into three groups, $A_i$, $B_i$ and $C_i$. The mean distance was 0·164 for group $A_i$ strains, 0·137 for group $B_i$, and 0·00 for group $C_i$ (four identical strains). Group $A_i$ included 21 strains, 18 of which were SEB+ ($P < 0{\cdot}01$); group $B_i$ included 13 strains, 11 of which were SEB+ ($P < 0{\cdot}05$); group $C_i$ included 4 strains, all of which were

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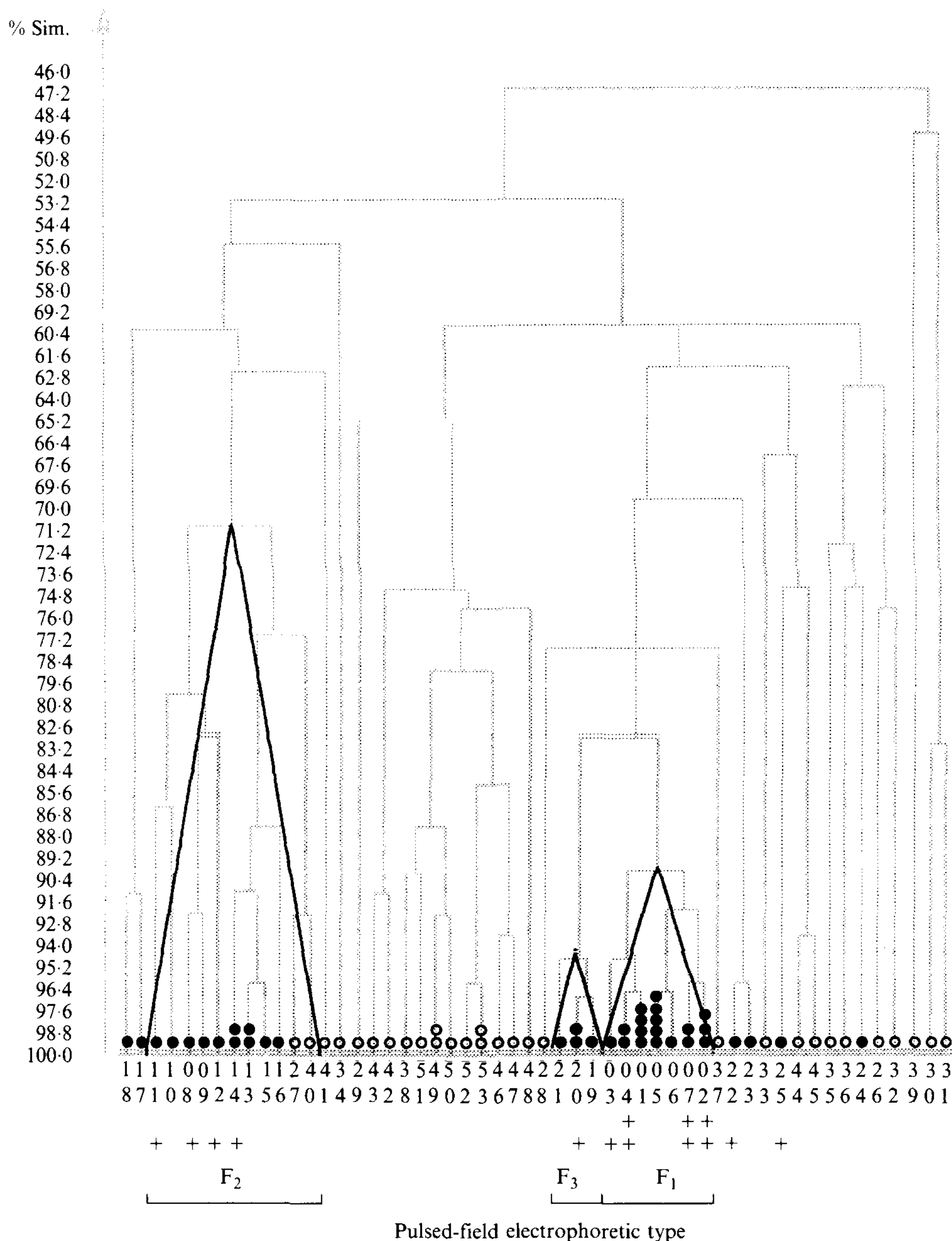


Fig. 3. Similarity dendrogram of *S. aureus* SEB+ (●) and *S. aureus* SEB− (○) strains. TS strains are represented +.

SEB+. Only 6 of the strains belonging to the other undefined groups were SEB+ ($P < 0{\cdot}01$).

*Ribotyping*

Each of the 28 ribotypes contained from 1–21 strains (Fig. 2). The 38 SEB+ strains (and not 39, as 1 strain was inadequately lysed) belonged to 8 ribotypes, whereas the SEB− strains belonged to 24 ribotypes. The dendrogram revealed

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two main groups within which were found most of the SEB+ strains: group $A_{rib}$, with 35 strains (28 SEB+, $P < 0{\cdot}01$) belonging to 5 ribotypes; and group $B_{rib}$, with 8 strains (6 SEB+) belonging to 4 ribotypes. The other strains (4 of which were SEB+, $P < 0{\cdot}01$) belonged to 19 ribotypes. The SEB− strains belonged to 24 ribotypes.

*Pulsed-field electrophoresis*

All the strains tested were divided into 52 electrophoretic types (Fig. 3). The dendrogram assigned the SEB+ strains to three main groups: $F_1$ (7 types) with 18 strains that were all SEB+ ($P < 0{\cdot}01$); $F_2$, with 11 of the 13 strains SEB+ ($P < 0{\cdot}01$); and $F_3$, with 4 SEB+ strains. The other strains, only 6 of which were SEB+, belonged to 32 different types ($P < 0{\cdot}01$).

*Strains associated with toxic shock*

Ten of 14 (71%) were penicillin-resistant, 3 (21%) were oxacillin-resistant, but 2 of them had MICs equal to 4 mg/l. Seven group-V TS+ belonged to phage group V (2 of which were V/+), 4 belonged to group II (1 was II/+), 1 belonged to group III/+, 1 belonged to a mixed group, and 1 was not typable. Ten strains could be classified in ribotype group $A_{rib}$, 2 in group $B_{rib}$, 2 others belonged to other ribotype groups. Seven belonged to zymogram group $A_i$, 4 to group $B_i$, and 1 to group $C_i$. The other two strains did not belong to any previously-defined group. The 7 $A_i$ strains belonged to the pulsed-field electrophoresis group $F_1$, the 4 $B_i$ strains to group $F_2$, 1 $C_i$ strain belonged to group $F_3$. The other two strains were not classified by the technique.

## DISCUSSION

In order to confirm or refute the hypothesis of a clonal origin of SEB+ strains, we compared 39 such strains with 30 SEB− strains, using 6 methods for phenotyping and genotyping: phage typing, serotyping, antibiotyping, zymotyping, ribotyping and pulsed-field electrophoresis. SEB+ strains differed from the SEB− control strains in that they were more homogenous when examined by ribotyping (38 SEB+ strains in 8 ribotypes versus 30 SEB− strains in 24 ribotypes), zymotyping (mean genetic heterogeneity diversity was 0·220 for SEB+, 0·295 for SEB−), and pulsed-field electrophoresis (39 SEB+ in 25 types versus 30 SEB− in 28 types).

However, they did not seem to constitute a unique clone: as shown in Table I, SEB+ strains were classified into 2 groups by ribotyping, group $A_{rib}$, with 28 strains, and group $B_{rib}$, with 6 strains. The isoenzyme ET divided $A_{rib}$ into two distinct profiles: types $A_i$ and $B_i$; the same distinction was observed by the pulsed-field DNA restriction profiles, types $F_1$ and $F_2$. Group $B_{rib}$ strains also were characterized by a distinct isoenzyme profile ($C_i$) as well as in pulsed-field electrophoresis ($F_3$). Both phage typing and serotyping discriminated similarly as regards phage group V and serotype b1/c1/o/p for strain $A_i$ and $F_1$, phage group II and serotype h2/263-2/l/m/o for strains $B_i/F_2$, and lastly phage group III or NT and serotype a4/a5/b1/c1//o/18 for strains $C_i/F_3$.

It is interesting to note that 5 oxacillin-resistant strains were detected in the

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Table 1. *SEB+ strains repartition in different groups: phage-type, antibiotype, ribotype, zymotype and pulse-field electrophoretype (PFE)*

| No. | Phage group | Antibiotype | Ribotype | Zymotype | PFE | |
|---|---|---|---|---|---|---|
| 4737 | V* | | Arib | $A_i$ | $F_1$ | |
| 4796 | V | | Arib | $A_i$ | $F_1$ | |
| 5406 | V/+ | S§ | Arib | $A_i$ | $F_1$ | |
| 5410 | V/+ | | Arib | $A_i$ | $F_1$ | TS†† |
| 6313 | V | *¶ | Arib | $A_i$ | $F_1$ | |
| 6740 | V/+ | * | Arib | $A_i$ | $F_1$ | TS |
| 860075 | V | | Arib | $A_i$ | $F_1$ | |
| 860167 | V | | Arib | $A_i$ | $F_1$ | TS |
| 860602 | V | | Arib | $A_i$ | $F_1$ | TS |
| 870113 | V | * | Arib | $A_i$ | $F_1$ | TS |
| 890240 | V | S | Arib | $A_i$ | $F_1$ | TS |
| 4275 | V | | Arib | $A_i$ | $F_1$ | |
| 5246 | M† | S | Arib | $A_i$ | $F_1$ | |
| 6506 | V/+ | | Arib | $A_i$ | $F_1$ | |
| 4997 | V/+ | | nd** | $A_i$ | $F_1$ | |
| 6499 | V | | Arib | $A_i$ | $F_1$ | |
| 5890 | V | | Other | $A_i$ | $F_1$ | TS |
| 6351 | V | * | Arib | $A_i$ | $F_1$ | |
| 4599 | II/+ | | Arib | $B_i$ | $F_2$ | TS |
| 850513 | II | | Arib | $B_i$ | $F_2$ | TS |
| 870449 | II | S | Arib | $B_i$ | $F_2$ | TS |
| 4262 | II | | Arib | $B_i$ | $F_2$ | |
| 6247 | II | | Arib | $B_i$ | $F_2$ | TS |
| 3433 | II | | Arib | $B_i$ | $F_2$ | |
| 6756 | II/+ | S | Arib | $B_i$ | $F_2$ | |
| 6837 | II | | Arib | $B_i$ | Other | |
| 6325 | II/+ | S | Other | $B_i$ | $F_2$ | |
| 6816 | M | S | Other | $B_i$ | $F_2$ | |
| 5664 | NT‡ | | Arib | $B_i$ | Other | |
| 4709 | II | | Arib | Other | $F_2$ | |
| 5414 | II | | Arib | Other | $F_2$ | |
| 870501 | NT | S | Other | Other | Other | TS |
| 5332 | M | S | Arib | Other | Other | |
| 6349 | III/+ | MRSA | Brib | $C_i$ | $F_3$ | TS |
| 4966 | III | MRSA | Brib | $C_i$ | $F_3$ | |
| 6477 | III/+ | MRSA | Brib | $C_i$ | $F_3$ | |
| 5231 | III | MRSA | Brib | Other | $F_3$ | |
| 5859 | NT | MRSA | Brib | $C_i$ | Other | |
| 5412 | M | | Brib | Other | Other | TS |

* V/+, group V and weak lysis by other phages.
† M, mixed group.
‡ NT, not typable.
§ S, susceptible to all antibiotics.
¶ *, borderline strains.
** nd, not determined.
†† TS, associated with toxic shock.

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specific group $B_{rib}/C_i/F_3$ and phage group III, and 4 weakly susceptible groups were in another subgroup, $A_{rib}/A_i/F_1$ and phage group V. The TS+ strains were assigned over all the subgroups.

A number of SEB+ strains were not classified into the main groups: for instance, strain 870501 belonged to no previously defined ribotype, zymotype or ET group, but was nonetheless not only susceptible to all the antibiotics but was also TS+. A number of atypical characters observed in phage typing and/or pulsed-field electrophoresis may be due to lysogenic phenomena. Such was the case also for strain 5332, which differed from other $A_{rib}$-group strains by the other associated characters; it was not only SEB+ but also SED+. Its marginal position may be explained by the fact that it belonged to another SED+ cell clone. Strain 5412, classified in group $B_{rib}$ with the methicillin-resistant *S. aureus* (MRSA), was susceptible to oxacillin but resistant to the aminoglycosides (apart from gentamicin and tobramycin), the macrolides (apart from pristinamycin) and to tetracycline.

Ribotyping with *Eco*R I restriction endonuclease allowed definition of cell clones at a high level. This property is undoubtedly due to the fact that the method is applicable to the taxonomy of staphylococci [23, 24]. It nonetheless discriminated between methicillin-susceptible and -resistant strains. Concordant results were yielded by the other markers: lysotyping, zymotyping, pulsed-field electrophoretyping, which confirms that SEB+ strains can be divided into three distinct groups $A_i/F_1$ on the one hand and $B_i/F_2$ on the other were regrouped under $A_{rib}$. In this case, interpretation of ribotypes was not evident, as ribotype groups 28, 1, 20, 2 and 7 were each distinct by 2 bands at the most, the other 10 fragments being of similar length. Under these conditions, they could not easily be considered as significantly different. Moreover, the wide variety of SEB-ribotypes suggests that strains with identical or close ribotypes may have a common parent. As regards the ribotype similarity of the two subgroups $A_i/F_1$ and $B_i/F_2$, this suggests that a member of the original clone may have undergone a considerable genetic modification that created another cell clone evolving in parallel, this modification in no way altering the ribotype that was highly insufficient to discriminate under species level. A possible relationship between the two clones can be confirmed only by comparing all the phenotypic and genotypic characters.

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