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Cytotoxin and Pyrogenic Toxin Superantigen Gene Profiles of *Staphylococcus aureus* Associated with Subclinical Mastitis in Dairy Cows and Relationships with Macrorestriction Genomic Profiles

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A set of 84 *Staphylococcus aureus* isolates collected from the milk of cows with subclinical mastitis in Asturias (a cattle region of Spain) and six control strains were tested for sequences of genes encoding hemolysins (*hla*, *hlb*, *hld*, *hlg*, and *hlg-2*), leukotoxins (*lukPV*, *lukM*, and *lukED*), toxic shock syndrome toxin (*tst*), and enterotoxins (*sea* to *see*, *seg* to *ser*, and *seu*) by conventional and multiplex PCR. It was found that 84, 83, 11, and 39 isolates carried some type of *hl*, *luk*, *tst*, or *se* gene, respectively, which were arranged in 14 exotoxin genotypes. All of the isolates were negative for *lukPV*, *hlg*, *sea*, *sed*, *see*, *sej*, *sek*, *sep*, *seq*, and *ser*. Two gene groupings could be related with pathogenicity islands—[*lukED*, *seg*, *sei*, *sem*, *sen*, *seo* ± *seu*] with Saβ-1 and [*tst*, *sec*, *sel*] with SaPIbov, present in 45 and 13.1% of the isolates, respectively—while 11.9% of them carried both islands. Only one contained *seb* (together with υSaβ-1), and another contained *seh* (together with *lukED*). The isolates were also analyzed by pulsed-field gel electrophoresis performed with SmaI. Thirty-nine SmaI profiles (similarity coefficient [*S*] = 0.94 to 0.21) were differentiated; 12, 1, and 10 of these, respectively, were generated by isolates presumptively carrying Saβ-1, SaPIbov, or both. Five SmaI profiles ($S \geq 0.8$) formed a cluster, which contained 20 and 10 isolates carrying one (υSaβ-1) or both islands. These data show the high frequency of genes encoding cytotoxins and pyrogenic toxin superantigens, their relationship with pathogenicity islands, and their distribution among a diversity of genetic types of *S. aureus* related to subclinical mastitis.

Staphylococcus aureus is a bacterium that colonizes and causes disease in mammalian hosts. It produces a wide variety of exoproteins that contribute to both colonization and the disease process (1, 7). Nearly all isolates secrete cytotoxins and enzymes (α-, β-, γ-, γ-variant, and δ-hemolysins; nucleases; lipases; hyaluronidase; and collagenase) whose main functions are to convert local host tissues into nutrients required for bacterial growth and also to promote spreading of the pathogen through the host body. Some isolates secrete one or more additional toxins, comprising leukotoxins (Luk) and pyrogenic toxin superantigens (PTSAgs), such as toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxins (SEs), and exfoliative toxins. PTSAgs have profound effects on the host immune system, both acute and long term. The former include food poisoning, caused by ingestion of SEs. Superantigens are thought to act as virulence factors by subverting immune responses and causing delays in the establishment of pathogen-specific immunity (1, 7, 16, 24, 27, 30, 34). γ-Hemolysin and leukotoxins (the classical Panton-Valentine [PVL], or LukS-PV-LukF-PV, and the new LukE-LukD and LukM-LukF′/PV) are two component toxins that kill erythrocytes and/or phagocytes by forming pores in cell membranes (7, 27, 30).

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Sequencing of complete or partial genomes from several *S. aureus* strains (3, 10, 14, 15, 17) has revealed the clustering of some exotoxin genes (encoding leukotoxins, TSST-1, and SEs) within chromosomal pathogenicity islands (SaPI or υSa) and that a single strain can carry more than one SaPI (3, 15, 29). One exotoxin gene cluster (*egc*) includes five SE genes [*seg*, *sei*, *seo*, *sen*, *sem*] and two pseudogenes (14) and was later identified in the genome of clinical strains as part of a genomic island, here called υSaβ-1 (3, 15, 29). More recently, an *egc* derivative encoding SEG, SEI, and SEN variants and the new SEU (which resulted from the insertion of 15 bp within one of the pseudogenes) has been identified (17).

S. aureus is recognized worldwide as a pathogen causing intramammary infections (mastitis) in cattle. Both leukotoxins and PTSAgs are supposed to play important roles in the initiation and/or exacerbation of mastitis (7, 27, 30). In addition, it has been proved that *S. aureus* is able to colonize milk and dairy products, in which some strains secrete toxins, including thermoresistant SEs. When foods contaminated with SEs are ingested, they can cause poisoning in humans, with fever, vomiting, nausea, abdominal pain, and diarrhea as the most frequent symptoms. In fact, the classical SEs (SEA to SEE), initially identified as serological types, were defined by their pyrogenic and emetic activities and only later categorized as superantigens. More recently, new SE types (SEG to SER and SEU) have been identified on the basis of sequence and structural similarities to previously described SEs, and there is also

experimental evidence for their superantigenic activity, but rarely for emetic activity (1, 7, 9, 14, 16, 17, 26).

To date, there is limited information about the prevalence of the recently identified toxin and exotoxin genes, including those encoding new leukotoxins and SEs, in *S. aureus* associated with infectious diseases in animals and human food poisoning. Taking this into account, the purposes of the present study were (i) to ascertain the frequency of genes encoding hemolysins (*hla*, *hlb*, *hld*, *hlg*, and *hlg-2*), leukotoxins (*lukS-PV*/*lukF-PV*, *lukM*, and *lukE-lukD*), TSST-1 (*tst*), and SEs (*sea* to *see*, *seg* to *ser*, and *seu*) in a set of 84 isolates of *S. aureus* collected from the milk of cows with subclinical mastitis in Asturias, a cattle region of Spain; (ii) to group the isolates according to exotoxin genotypes (EGs) and to establish possible gene clusters and their association with SaPIs; (iii) to discriminate isolates into genomic types defined by macrorestriction–pulsed-field gel electrophoresis (PFGE) performed with SmaI and cluster analysis, by which means we aimed to determine the most frequent genomic types with the potential for colonizing cow mammary glands and cow milk; and (iv) to correlate EGs and SaPIs with genomic types, thus tracing the dispersion of genes associated and not associated with SaPIs among genomic types.

MATERIALS AND METHODS

***S. aureus* isolates.** The *S. aureus* isolates used in this study were recovered from milk samples from 84 cows (one isolate per cow) with subclinical mastitis in Asturias from 2003 to 2004. Asturias is a northern Spanish region covering an area of ~10,565 km^2 and has ca. 4,700 dairy cattle herds, each including between 4 and 200 cows. For the control of mastitis, recounts of somatic cells in pooled milk samples from each herd are routinely performed four times per month using a Fossomatic 5000 autoanalyzer (FOSS A/S, Hillerød, Denmark). Mastitis is suspected when counts of ≥250,000 cells/ml are obtained and a decrease in milk production and/or moderate inflammation of the udder is observed. Milk samples from each cow of a suspected herd are independently assayed by the California Mastitis Test using commercial kits (ImmuCell, Portland, Maine, or Westfalia-Surge Inc., Naperville, Ill.). The results are visually scored on the basis of gel formation. Trace and weak-positive reactions are taken as indicators of subclinical mastitis. Infection is confirmed on the basis of elevated white cell numbers in the milk sample (>400,000 leukocytes/ml) and/or decrease in milk quality and isolation of pathogenic bacteria. For isolation of *S. aureus*, 20 μl of milk were spread and incubated at 37°C for 24 h on plates of Columbia blood agar (bioMérieux S.A., Marcy-l'Etoile, France). Identification of presumptive *S. aureus* colonies was carried out by the following assays: Gram staining, coagulase production (the Staphylase test) and thermonuclease production on DNase medium (both from Oxoid Diagnostic Reagents, Basingstoke, United Kingdom), and hemolytic activity on Columbia blood agar. They were also biochemically identified by the API Staph system (bioMérieux S.A). In addition, the 84 *S. aureus* isolates were tested for production of five classical PTSAgs by reverse passive latex agglutination using the TST-RPLA kit for TSST-1 and the SET-RPLA kit for SEA to SED (Oxoid Diagnostic Reagents). Six strains, each the prototype of a specific toxin (see Table 2), were used as controls in different experiments.

DNA isolation and PCR procedures. Genomic DNA isolation and detection of exotoxin-encoding genes (by conventional and multiplex PCR) were achieved as previously reported (20). In all PCR assays, each isolate was tested at least twice, and positive and negative controls were always included. Primers previously described for *hla*, *hlb*, *hld*, *hlg*, *hlg-2*, *lukS-PV*/*lukF-PV* (*lukPV*), *lukM*, *lukE-lukD* (*lukED*), *sei*, and *sen* (generating amplicons of 209, 309, 111, 535, 390, 433, 780, 269, 576, and 680 bp, respectively) (13); *sea* to *see* and *sej* (generating amplicons of 566, 424, 315, 401, 512, and 648 bp, respectively) (20); *seh* and *seg* (with amplicons of 494 and 683 bp) (19); *sek* and *seg* (with amplicons of 278 and 285 bp) (35); and *seu* (141-bp amplicon) (17) were used. Primers for other genes were designed in our laboratory from the sequences deposited in EMBL: *tst* (tst-1, CATCTACAAACGATAATATAAAGG, and tst-2, CATTGTTATTTTCCAA TAACCACCCG; accession number AP003135), *sel* (sel-1, AATATATAACTA GTGATCTAAAGGG, and sel-2, TATGGAATACTACACACCCCTTATA; accession number AP003135), *sem* (sem-1, ATGCTGTAGATGTATATGGTC TAAG, and sem-2, CGTCCTTATAAGATATTTCTACATC; accession number AP003363), *seo* (seo-1, TGTAGTGTAAACAATGCATATGCAAATG, and seo-2, TTATGTAAATAAATAAACATCAATATGATGTC; accession number AP003363), *sep* (sep-1, TTAGACAAACCTATTATCATAATGG, and sep-2, TATTATCATGTAACGTTACACCGCC; accession number NC 002745), and *ser* (ser-1, AACCAGATCCAAGGCCTGGAG, and ser-2, TCACATTGTAGT CAGGTGAACTT; accession number AB075606), which generated amplicons of 481, 359, 473, 722, 276, and 700 bp, respectively. In multiplex PCR, different sets of primers were assayed with the control strains. On the basis of amplicon profile and reproducibility, nine primer sets were selected to test the 84 mastitis isolates: set 1 (*hla*, *hlb*, and *hld*), set 2 (*hlg* and *hlg-2*), set 3 (*lukPV*, *lukM*, and *lukED*), set 4 (*sea*, *seb*, and *sec*), set 5 (*sed*, *see*, and *sej*), set 6 (*seg*, *seh*, and *sei*), set 7 (*sem*, *sen*, and *seu*), set 8 (*sel*, *seo*, and *sep*), and set 9 (*seq*, *ser*, and *tst*). The *sek* primers did not generate amplicons in the different combinations tested. For this reason, they were only tested alone.

Macrorestriction-PFGE analysis. Total DNA from each *S. aureus* isolate was analyzed by macrorestriction-PFGE performed with SmaI by means of the CHEF-DRIII SYS220/240 (Bio-Rad Laboratories S.A., Madrid, Spain), basically using the consensus protocol (22). SmaI banding profiles were visually analyzed, and the presence or absence of each band was recorded. Those showing one or more mismatched bands were considered different and numbered as S80 to S92 (isolates containing PTSAg genes) and S101 to S125 (isolates without PTSAg genes). S1 to S79 have been used in our laboratory to label SmaI profiles generated by *S. aureus* from other origins. Relationships between SmaI profiles were established on the basis of the number of mismatched bands (22, 33), and the genetic similarity was determined by the unweighted pair method with arithmetic averages and Jaccard's similarity coefficient (*S*) in the software Program MVSP version 3.1 (Multivariate Statistics Package for PCs; RockWare Inc.). The discrimination index (*DI*) (i.e., the probability that two unrelated isolates obtained from the population would be placed into different SmaI profiles) was calculated using Simpson's index of diversity (31).

RESULTS

Phenotypic features and exotoxin genotypes of *S. aureus* isolates associated with subclinical mastitis in cows. The *S. aureus* isolates were recovered from 84 milk samples (each from a different cow with subclinical mastitis) spread on blood agar, where they produced creamy grayish-white or yellow colonies with distinct zones of hemolytic activity. In addition, they were positive in catalase, coagulase, and DNase tests. From each sample, one isolate was selected for further study (n = 84). When tested by reverse latex agglutination, none of the 84 isolates was positive for SEA or SED, only one produced SEB, and 11 produced both SEC and TSST-1. Next, detection of 27 exotoxin genes by conventional and multiplex PCR revealed that all isolates were negative for *lukPV*, *hlg*, *see*, *sej*, *sek*, *sep*, *seq*, and *ser*. The remaining exotoxin genes were found with different frequencies and EGs (Table 1). A total of 14 EGs were distinguished (*DI* = 0.8). The 84 isolates were positive for *hla* and *hlg-2*, and 81 and 76 of them also contained *hld* and *hlb*, respectively. All except one isolate were positive for *lukED*, and 42 also harbored *lukM*. The latter gene was more frequently detected in isolates carrying PTSAgs than in those lacking PTSAGs (34 versus 8). The single SEB- and the 11 [TSST-1, SEC]-positive isolates, and only these, were also positive for *seb* and [*tst*, *sec*, *sel*], respectively; 38 isolates proved to contain [*seg*, *sei*, *sem*, *sen*, *seo*], and 32 of these also contained *seu*. Since these genes are characteristic of *egc* clusters, they will be referred to here as either *egc-1* (*seu* negative) or *egc-2* (*seu* positive). The single *seb* isolate also carried *egc-1*, while 10 out of the 11 [*tst*, *sec*, *sel*] isolates contained *egc-2*. Finally, a single *seh* isolate was negative for all other PTSAg genes tested. As indicated above, these results were obtained by both single and multiplex PCR, and representative examples of the

TABLE 1. Exotoxin genotypes and macrorestriction genomic profiles of *S. aureus* collected from milk samples from cows with subclinical mastitis

EG (no. of isolates)	Cytotoxin(s)[a]	PTSAg superantigens[b]	SmaI macrorestriction analysis	
			Profile	Cluster no.
EG1 (10)	*hlb*, *hld*, *lukED*, *lukM*	*tst*, *sec*, *sel*, [*seg*, *sei*, *sem*, *sen*, *seo*, *seu*]	S80	3
			S81	2
			S82	5
EG2 (22)	*hlb*, *hld*, *lukED*, *lukM*	[*seg*, *sei*, *sem*, *sen*, *seo*, *seu*]	S83, S91, S92	1[c]
			S84	4
			S82	5
			S80	8
			S89	2
EG3 (1)	*hlb*, *hld*, *lukED*, *lukM*	[*seg*, *sei*, *sem*, *sen*, *seo*]	S83	1
EG4 (1)	*hlb*, *hld*, *lukED*, *lukM*	*tst*, *sec*, *sel*	S86	1
EG5 (6)	*hlb*, *hld*, *lukED*, *lukM*		S121	2
			S103, S119, S123, S125	1[c]
EG6 (1)	*hld*, *hld*, *lukED*	*seb*, [*seg*, *sei*, *sem*, *sen*, *seo*]	S85	1
EG7 (3)	*hlb*, *hld*, *lukED*	[*seg*, *sei*, *sem*, *sen*, *seo*]	S87	1
			S90	2
EG8 (1)	*hld*, *lukED*,	[*seg*, *sei*, *sem*, *sen*, *seo*]	S88	1
EG9 (1)	*hlb*, *hld*, *lukED*	*seh*	S93	1
EG10 (28)	*hlb*, *hld*, *lukED*		S101, S106, S120	2[c]
			S103	4
			S104	7
			S92, S102, S105, S108, S111, S112, S113, S114, S115, S117, S118	1[c]
EG11 (2)	*hld*, *lukED*, *lukM*		S122	2
EG12 (3)	*hlb*, *lukED*		S103, S107, S124	1[c]
EG13 (4)	*hld*, *lukED*		S109, S116, S119, S120	1[c]
EG14 (1)	*hld*		S110	1

[a] All subclinical mastitis isolates were positive for the *hla* and *hlg-2* genes.
[b] [*seg*, *sei*, *sem*, *sen*, *seo*] and [*seg*, *sei*, *sem*, *sen*, *seo*, *seu*] have been found associated in gene clusters *egc-1* and *egc-2*, respectively (see the text).
[c] Only one or two strains (as indicated) belonged to each profile.

latter are shown in Fig. 1. The EGs of the control strains are indicated in Table 2. The six strains were *hla* and *lukED* positive, and three of them also contained *egc-2*.

PFGE-genomic macrorestriction analysis of toxigenic *S. aureus* isolates. All subclinical mastitis isolates could be assigned to distinctive profiles when tested by PFGE-macrorestriction performed with SmaI. In order to define profiles, only those fragments ranging between ca. 40 and 700 kb were considered. In total, 39 SmaI profiles could be differentiated, with the six control strains generating six additional SmaI profiles (Fig. 2). This procedure yielded a *DI* of 0.956 for the mastitis isolates ($n = 84$) and a *DI* of 0.961 if the six reference strains were also included ($n = 90$). The distribution of isolates among SmaI profiles is compiled in Table 1.

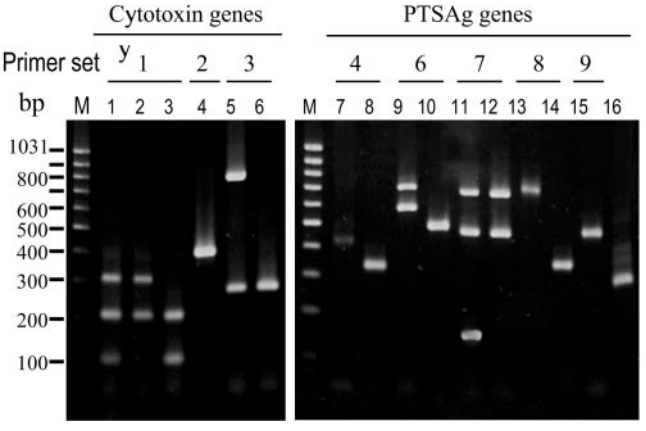

FIG. 1. Screening of exotoxin genes by multiplex PCR in representative *S. aureus* isolates associated with subclinical mastitis. Lanes M, lambda ladder PFGE markers (New England Biolabs). Lanes 1 to 15, amplicon profiles generated by mastitis isolates using the primer sets indicated at the top. Lane 16, amplicon generated using the *sek* primers. Each amplicon profile was included in different EGs (Table 1).

A dendrogram was constructed on the basis of coefficients of similarity between SmaI profiles (Fig. 3), and it showed considerable variation (0.94 to 0.21). At a cutoff point of $S = 0.7$, seven clusters (C1 to C7) were revealed, with two of them (C1 and C2) grouping SmaI profiles from PTSAg-positive isolates (in which several cytotoxin genes were also detected). C2 included five profiles (S80 to S84, with ≤6 mismatched fragments), represented by PTSAg isolates belonging to three genotypes: EG1 ([*tst*, *sec*, *sel*] and *egc-2*; 10 isolates), EG2 (*egc-2*; 18 isolates), and EG3 (*egc-1*; 1 isolate). In contrast, only two profiles (S88 and S90) represented by *egc-1*-containing genotypes (EG8 and EG7, respectively) fell within C1. In addition, PTSAg isolates were also assigned to several nonclustered SmaI profiles (at the established cutoff point; see above). Thus, profiles S85, S86, and S93 were represented by the single EG6, EG4, and EG9 isolates, respectively. Four other profiles (S87, S89, S91, and S92) were generated from isolates containing *egc-1* (EG7) or *egc-2* (EG2) and by one *egc*-negative isolate (EG10; S92) (Table 1). The remaining 25 profiles (S101 to

TABLE 2. Exotoxin genotypes of *S. aureus* control strains

Strain[a] and toxin prototype	Cytotoxin genes	PTSAg genes
CECT 976 (ATCC 13565), SEA, SED	*hla*, *hlb*, *hld*, *hlg-2*, *lukED*	*sea*, [*sed*, *sej*, *ser*][b]
CECT 4459; SEB	*hla*, *hld*, *hlg-2*, *lukED*	*sea*, [*seb*, *sek*, *seq*][c]
CECT 4465 (ATCC 19095); SEC	*hla*, *hlb*, *hld*, *hlg-2*, *lukED*	[*sec*, *sel*, *sem*],[d] [*seg*, *sei*, *sem*, *sen*, *seo*, *seu*][e]
CECT 59 (ATCC 9114); PVL	*hla*, *hld*, *hlg*, *lukED*, *lukPV*	[*seg*, *sei*, *sem*, *sen*, *seo*, *seu*][e]
CNM 3194/98; TSST-1	*hla*, *hlb*, *hld*, *hlg*, *lukED*	*tst*,[f] [*seg*, *sei*, *sem*, *sen*, *seo*, *seu*][e]
NCTC 8325; LukE-LukD	*hla*, *hld*, *hlg-2*, *lukED*	

[a] CETC, Colección Española de Cultivos Tipo, ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CNM, Centro Nacional de Microbiología.
[b] Genes carried on plasmids (16, 26) are indicated in brackets.
[c] Gene cluster associated with SaPI3 (24, 29) are indicated in brackets.
[d] Gene cluster associated with SaPI4 (24, 29) are indicated in brackets.
[e] Variant of the *egc* cluster (here called *egc-2*) including the *seu* gene (17) are indicated in brackets.
[f] Gene associated with SaPI2 (24, 29).

S125) were represented by 43 isolates that were negative for PTSAg genes, assigned to six EGs (EG5 and EG10 to -14). They showed high genetic heterogeneity (S = 0.88 to 0.21) (Fig. 3), although some of them could be grouped into five additional clusters (C3 to C7).

DISCUSSION

Results derived from the present research into *S. aureus* isolates recovered from milk samples from cows with subclinical mastitis will be discussed in terms of the relevance of the presence of many different virulence genes in both the pathogenesis of bovine mastitis and food safety. It is noteworthy that all except one isolate carried some type of leukotoxin gene, but only half were positive for *lukM*, the gene that encodes the most active leukotoxin for polymorphonuclear leukocytes of ruminants found in mastitis isolates (27). In addition, about half of the isolates (including both *lukM*-positive and -negative isolates) carried a number of PTSAg genes, ranging from one to nine. These results are compatible with the hypothesis that the maintenance of PTSAgs is useful for the bacterium, as they may inhibit the efficiency of the immune response of the cow (8). However, our failure to detect PTSAg genes in the other half of the isolates argues against an essential role in subclinical mastitis. Alternatively, *S. aureus* might still produce previously unidentified PTSAgs that escaped detection by the techniques employed. Although the tested isolates were recovered from cows diagnosed with subclinical mastitis, the possibility that they could simply be members of the common microbiota of the cow should also be considered. In spite of this, the possession of multiple virulence genes (and the fact that virulence is multifactorial) supports a strong potential of the isolates to cause not only subclinical mastitis in their hosts but also severe illness, including clinical mastitis. Moreover, the presence of toxigenic *S. aureus* in milk could also represent a

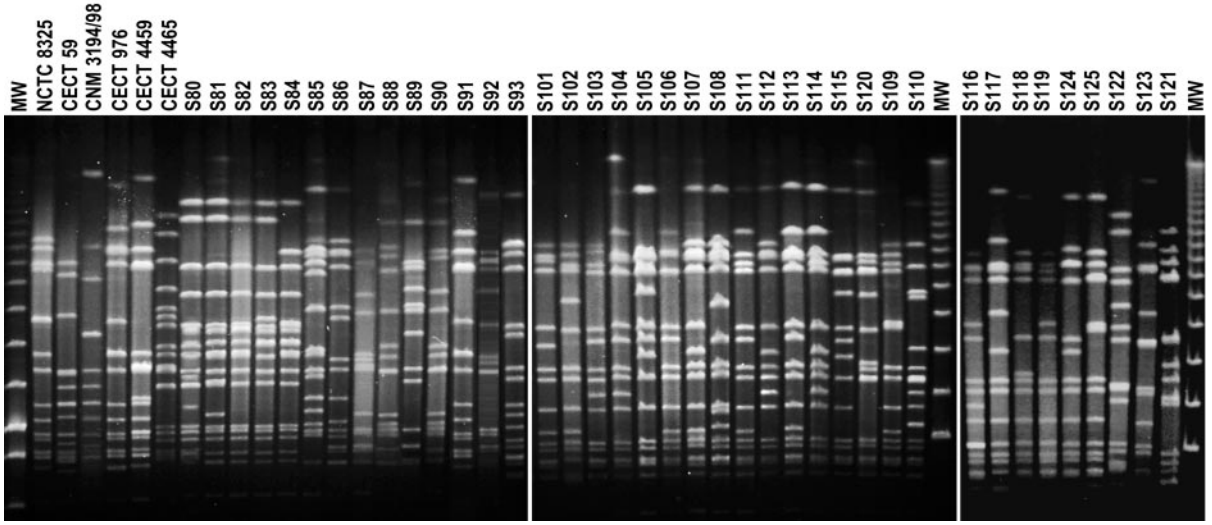

FIG. 2. Macrorestriction genomic profiles generated by SmaI in *S. aureus* control strains and isolates associated with subclinical mastitis. Lanes MW, lambda ladder PFGE markers (New England Biolabs). Lane NCTC 8325, quality control strain in PFGE assays. Fragment sizes from top to bottom in (kilobases) are 674, 361, 324, 262, 257, 175, 135, 117, 80, 60 and <60 (the 208-kb fragment present in the PFGE profile shown in reference 20 is lacking). NCTC 8325 was also used as a LukE-LukD prototype and an SE-TSST negative control. The next five lanes show the SmaI profiles generated by the prototype strains of PVL (CECT 59), TSST-1 (CNM 3194/98), SEA and SED (CECT 976), SEB (CECT 4459), and SEC (CECT 4465), respectively. Lanes S80 to S92 and S101 to S125 correspond to profiles generated by isolates positive and negative, respectively, for PTSAg genes (except S92, which includes both types). The relationships between EGs and SmaI profiles are compiled in Table 1.

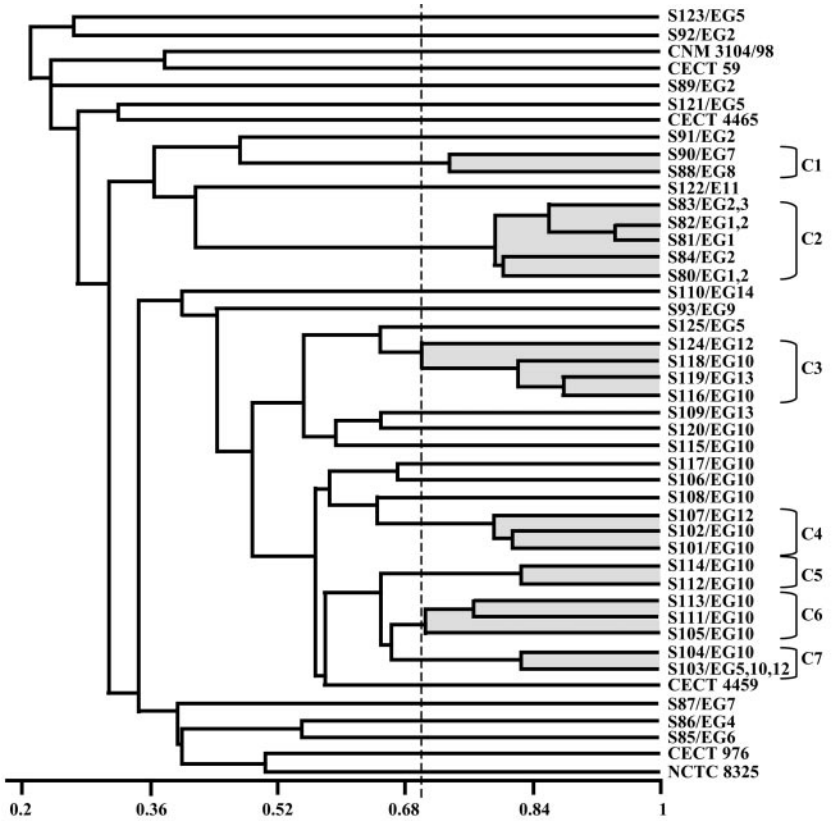

FIG. 3. Dendrogram showing genetic relatedness among SmaI macrorestriction fragment profiles generated from *S. aureus.* Cluster analysis was performed by the Jaccard similarity coefficient and the unweighted pair group method. Branches CECT 59, CECT 976, CECT 4459, CECT 4465, CNM 3194, and NTCC 8325 correspond to exotoxin prototype strains (Table 2). Branches S80 to S92 and S101 to S125, respectively, correspond to isolates associated with subclinical mastitis that were positive and negative for PTSAg genes (except S92, which includes both). At a cutoff point of $S = 0.7$, seven clusters (labeled C1 to C7) and 24 branches (6 corresponding to the prototype strains) were found. EG, exotoxin genotypes of isolates associated with subclinical mastitis. The numbers of isolates falling into each cluster and branch are compiled in Table 1.

serious problem for food security, if milk containing SEs (frequently with a normal appearance) reaches the human food chain, either directly or through dairy products. In fact, poisoning outbreaks caused by SE-contaminated milk are not rare. An outstanding example was an outbreak due to low-fat milk that occurred in Japan in 2000, with 13,420 cases recorded (2).

Our data on the frequency of hemolysin and leukotoxin genes are in agreement with those reported by Rainard et al. (27) showing that all isolates analyzed from a collection of *S. aureus* recovered from ruminants with mastitis harbored at least the genes for γ-hemolysin and LukE-LukD. However, they differ from those of another study (30) with isolates causing clinical and subclinical mastitis in which only 55 and 41%, respectively, produced leukotoxins. With regard to PTSAg genes, *seb* and *seh* were each found in one isolate, while the remaining genes had a wider distribution and appeared in three different clusters: [*tst*, *sec*, *sel*], *egc-1*, and *egc-2*, found in 13.1, 7.1, and 38.1% of the isolates, respectively. The presence of nine PTSAg genes encoding two classical (TSST-1 and SEC) and seven new (SEL, SEG, SEI, SEM, SEN, SEO, and SEU) superantigens in 11.9% of the isolates is noteworthy. However, a correlation of the number of PTSAg genes with the severity of mastitis has not been recorded. The classical PTSAgs found in *S. aureus* isolates from Asturias (SEB and TSST-1–SEC) coincided with those reported for mastitis isolates collected in Ireland, the United States, and Japan (9, 23). However, they differed from data available for Italian and Korean mastitis isolates, where SED or SEA, respectively, were the most frequent toxins (5, 18), and for isolates recovered in Brazil, 65% of which produced one or several toxins but only 3.2% of which produced TSST-1–SEC (4). In the present study, classical and new *se* genes were found in 12 versus 40 isolates, respectively (ca. 1:3 proportion), and similar or even greater differences have been reported in previous screenings of isolates from a different origin in which some of the new *se* genes were included (14, 21, 25, 26, 28). This is notable, since it has been recently reported that there are large gaps in the capacity of human serum samples to neutralize *S. aureus* PTSAgs and that the *egc*-encoded superantigens are neutralized by human sera much less efficiently than classical PTSAgs (12). It is also notable that, in the present work, only 6 out of 38 isolates found to contain *egc* clusters (15.8%) were *seu* negative, while the remaining 32 (84.2%) were *seu* positive. Thus, in our series, the incidence of *egc* including the *seu* gene (i.e., *egc-2*) was much higher than that previously reported: 16.6% (17). Discrepancies between results from different countries as to the frequency of leukotoxin genes, PTSAg genes, and *egc* are not

unexpected, since (i) these genes are associated with mobile (and therefore variable) genetic elements, including SaPIs, plasmids, and phages; (ii) different strains could have gained, and maintained over time, a variable number of such elements; (iii) in agreement with the previous points, dairy cows from different geographic areas could have been colonized by strains with different virulence genotypes; and (iv) all or some of these strains, under certain conditions, may be able to invade the skin and mucous membranes of the host, causing severe illness.

When the gene clusters found in this study were compared with information available in the literature, the following noteworthy observations were made. (i) Clustering of *tst*, *sec*, and *sel* genes (here reported in 13.1% of the isolates) was initially described in SaPIbov (υSa2), a genomic island found in *S. aureus* of bovine origin (10). However, this gene association is also present in several islands (SaPIn1/SaPIm1 and SaPIn3/SaPIm3, belonging to the υSa2 and υSa4 families) that were identified in the genomes of human isolates (3, 15, 24, 29). (ii) Sequencing of the complete genome of *S. aureus* NCTC 8325 (here included as a control strain) and of human methicillin-resistant isolates revealed the presence of *lukED* in two chromosomal islands: υSaβ-1, which also carries *egc-1*, and υSaβ-2 (3, 29). Experimental data reported in this work support the presence of υSaβ-1 in six milk isolates and suggest the existence of a υSa variant, harboring *egc-2* instead of *egc-1*, in 32 *seu*-positive isolates. Our results also suggest the presence of υSaβ-2, or another element containing *lukED*, in all but one of the PTSAg-negative isolates.

PFGE-macrorestriction analysis has been reported as the "gold standard" method for typing bacteria. In fact, the procedure has already been used to discriminate *S. aureus* isolates from different human, animal, and food samples (6, 18, 21–23, 32, 33). In this work, an SmaI-PFGE procedure, previously harmonized for typing methicillin-resistant *S. aureus* isolates (22), was applied to type the 84 mastitis isolates. In this way, they were discriminated into 39 SmaI genomic profiles, which showed broad genetic heterogeneity ($S = 0.94$ to 0.21). The 44 isolates containing PTSAg genes generated 16 SmaI profiles, only one of which (S92) also contained a non-PTSAg isolate. It is noticeable that five SmaI profiles formed a cluster (C2; $S \geq 0.80$) that included 38.1% of the isolates presumably carrying the υSaβ-1 variant, and some of them also carried SaPIbov. Accordingly, a wide variety of genomic types of toxigenic *S. aureus* could be part of the normal microbiota of cows and also be involved in bovine subclinical mastitis in Asturias. Mastitis (clinical and subclinical) generates important losses in dairy industries in the region and, as previously indicated, can be regarded as a risk factor for food poisoning in humans.

Typing results, together with the possible clustering of PTSAg and leukotoxin genes within υSaβ-1 islands (which harbor transposase genes, indicating that transposons may have been the origin of these islands) (3, 15, 29), support the following hypotheses. (i) Horizontal transmission of the island(s) among strains of different origin; in fact, subclinical mastitis isolates generating highly different macrorestriction genomic profiles ($S = 0.2$ to 0.9) contained seven or eight genes associated with Saβ-1 islands. A similar situation was found in *S. aureus* isolates from healthy human carriers analyzed in our laboratory (unpublished results). Notably, *egc-1* or *egc-2* was identified in a high proportion of the human isolates, which included producers and nonproducers of classical PTSAgs. However, none of the isolates displayed a PTSAg gene profile consistent with SaPIbov (references 11 and 21 and unpublished data). A wide distribution of *egc* (or its associated *seg-sei* genes) has also been reported for human and food-borne *S. aureus* isolates in other works (12, 14, 21, 25, 28). (ii) Clustering of leukotoxin and superantigen genes may be beneficial for *S. aureus* when colonizing and/or invading human and animal tissues and organs, apart from their involvement in disease (14). The same is expected for the insertion of different SaPIs into the genome of a single strain. The fact that 45 and 11.9% of our subclinical mastitis isolates, respectively, contained genes associated with Saβ-1 (without excluding the concomitant presence of Saβ-2) and with υSaβ-1 together with SaPIbov is then relevant. (iii) Theoretically, the accumulation of *se* genes in a single strain could be related to the amount of SEs secreted in foods and therefore to the severity of food poisoning. It should be remembered that SEs act not only as superantigens but also as potent gastrointestinal toxins and that these functions are located in two separate domains (1, 7, 16). Finally, the high frequency of *egc* clusters, or their associated *seg-sei* genes, highlights the requirement in prospective or future studies of food poisoning caused by *S. aureus* to screen the highest possible number of SEs, or their *se* genes, including *egc* genes. For this, multiplex PCR could be an accurate and relatively simple procedure.

In this context, it should be borne in mind that the screening of a large number of gene sequences and isolates by conventional PCR is more time-consuming and expensive than multiplex PCR. In the present work, the screening of 27 exotoxin genes at least twice in 84 subclinical mastitis isolates plus six control strains required >5,000 conventional PCRs. The same analysis was performed with less than half the number of multiplex reactions. Therefore, the latter option represents an important saving in time and resources. However, future screenings would require an improvement of the multiplex PCR protocols reported here in order to reduce the number of reactions and to limit false-negative results (here detected by comparison with the results of conventional PCR). For this purpose, the design and evaluation of new primers and primer combinations would allow the simultaneous and reliable screening of a higher number of genes. In fact, multiplex PCR using more than four primer pairs has been successfully achieved in previous work related to the subject (19–21), although the number of screened genes was always lower than that of the present study. Finally, it is also important to ascertain the reasons for failure of *sek* amplification when attempted in conjunction with that of other genes.

In conclusion, the following findings in relation to the aims of this study can be highlighted. (i) Nearly all *S. aureus* isolates related to subclinical bovine mastitis carried genes encoding hemolysins and leukotoxins, but only half of them carried genes encoding PTSAgs. In total, the exotoxin genes could be grouped into 14 profiles, or EGs. (ii) Most of the PTSAg genes were located on two pathogenicity islands (SaPIbov and υSaβ-1), which appeared either alone or coexisting in the same isolate. (iii) SaPIbov was mainly associated with organisms falling into one SmaI cluster, whereas υSaβ-1 appeared to be dispersed in this cluster, as well as in other clustered and nonclustered isolates, showing it to be a highly ubiquitous

element. (iv) A wide variety of genomic types of toxigenic *S. aureus* could be regarded as both possible causal agents of clinical disease and risk factors for food poisoning.

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