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Staphylococcus aureus agr Genotypes with Enterotoxin Production Capabilities Can Resist Neutrophil Bactericidal Activity

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Staphylococcus aureus **pathogenicity is mainly due to the production of a number of secreted and cell surface-associated proteins under the regulation of the *agr* gene. A region of the *agr* gene was used to subgroup *S. aureus* strains according to restriction fragment length polymorphisms. Additionally, strains were subtyped according to the coagulase gene in order to strengthen discriminatory power. Virulence capabilities of *agr* genotype subgroups were evaluated using an in vitro neutrophil bactericidal assay, which showed that prevalent genotypes were significantly better at evading this primary host defense. Multiplex PCR was then used to detect enterotoxin genes among the genotype subgroups in order to determine possible virulence candidates that enable strains to combat neutrophil killing. The prevalent genotype strains were found to possess higher production capabilities for enterotoxin A than did low-prevalence strains. The significance of enterotoxin A production capabilities in affecting pathogenicity of *S. aureus* strains was evaluated and found to have a profound effect on neutrophil killing abilities. The use of a large epidemiological database as a tool for subgrouping strains with varying degrees of pathogenicity has allowed the identification of relevant and previously undefined virulence factors that affect a pathogen's capability to overcome host immune defenses.**

Staphylococcus aureus is a gram-positive bacterium that has remained a persistent pathogen, causing such infections as endocarditis, meningitis, and toxic shock syndrome in humans. *S. aureus* also is the leading cause of intramammary infections (mastitis), especially in dairy animals, from whose milk it is frequently isolated (38). Neutrophils are the principle line of defense during the initial stages of mastitis, and the ability of these cells to phagocytize and kill invading bacteria is critically related to the establishment of new intramammary infections (26). Therefore, any bacterially derived component that may compromise neutrophil function would constitute an important virulence factor in the pathogenesis of *S. aureus* mastitis. Although a number of different virulence factors involved in the pathogenesis of *S. aureus* mastitis have been identified (38), the differential expression of these factors as it relates to field strain prevalence of *S. aureus* genotypes has not been investigated. A better understanding of the epidemiology of *S. aureus* mastitis as it pertains to virulence will provide insight concerning important host-pathogen interactions during the pathogenesis of disease.

Subtyping is an important tool for epidemiologic investigation of bacterial infections. In the past decade, numerous molecular techniques such as multilocus enzyme electrophoresis, phage typing, plasmid DNA restriction patterns, random amplified polymorphic DNA ribotyping, and coagulase genotyping have proved useful in identification and comparison of *S. aureus* isolates in epidemiological studies (7, 21, 29, 35, 36). However, very few studies have identified *S. aureus* isolates by the gene polymorphisms among important virulence-related genes. Among the virulence-related genes in *S. aureus*, we were particularly interested in the accessory gene regulator (*agr*), which has been shown to regulate the synthesis of many virulence factors during bacterial growth (5, 25). The *agr* system coordinately down-regulates the production of cell wall-associated proteins and up-regulates secreted proteins at late to stationary growth phase in vitro (16, 24, 25, 27). The *agr* locus encodes a two-component signal-transducing system consisting of two divergent transcription units driven by promoters P2 and P3 (15). The P3 operon encodes the transcript for RNAIII, the effector of the *agr* response, while the P2 operon contains transcripts for four open reading frames designated *agrA*, *-B*, *-C*, and *-D* (6). *agrB* and *-D* generate an autoinducing peptide that acts as an activating ligand for *agrC*. Interestingly, Ji et al. (15, 16) have shown that variations in the gene sequences of *agrB* and *-D* result in variation of the autoinducing peptide that, in turn, causes differences in the activation of strains by one another (15, 16). In contrast, mutations of wild-type *S. aureus* strains resulting in *agr* deletions reduced persistence of infection, exotoxin synthesis, and binding capabilities and decreased intracellular growth of these strains (3, 11, 37), suggesting that *agr* itself is an important virulence gene in *S. aureus*.

The aim of this study was to identify potential *S. aureus* virulence factors based on their unique expression in predominant field strains isolated from clinical cases of mastitis. In this study, we developed a genotyping method for *S. aureus* strains based on *agr* gene polymorphisms. We showed that enterotoxin production capabilities were more pronounced in the prevalent *agr* genotypes that were more resistant to neutrophil bactericidal activities than were the low-prevalence genotypes. The ability of enterotoxin A to directly modify neutrophil function suggests an important role of these toxins in the pathogenesis of mastitis.

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MATERIALS AND METHODS

Bacterial isolates. The *S. aureus* strains used in this study included RN6390B, a wild-type *agr*$^+$ strain (25); RN6911, an *agr* mutant (25); SA 502A (ATCC 27217); and 255 field isolates. *S. aureus* field isolates were collected from clinical mastitis bovine milk samples from the Czech Republic (n = 10), France (n = 34), Korea (n = 165), and several locations within the United States (n = 46), (including Indiana, Kentucky, Louisiana, Minnesota, New York, Pennsylvania, Tennessee, Washington, and Wisconsin). The variety of geographical locations from which isolates were collected provided control for regional differences in herd management and herd differences that result in variations in host resistance to disease. All isolates were stored in Trypticase soy broth with 15% glycerol at −70°C until needed. Isolates were cultured on Trypticase soy agar with 5% sheep blood (BiMed, St. Paul, Minn.) for identification based on colony morphology, hemolysis, Gram stain, and acetoin and catalase production. Coagulase production by isolates was determined in a tube test using 0.5 ml of citrate-stabilized rabbit plasma. One colony from an overnight culture on Trypticase soy agar–5% sheep blood was inoculated into a plasma-containing tube and incubated at 37°C. A positive test was determined by clot formation after 1, 4, or 24 h. To differentiate *S. aureus* from coagulase-positive *S. hyicus* and *S. intermedius*, the acetoin test (Voges-Proskauer test) was used as outlined previously (1). All isolates with a questionable acetoin test result were further identified by the API Staph system (bioMerieux Vitek, Hazelwood, Mo.).

Bacterial DNA lysates. Bacterial DNA lysates were prepared from 1 ml of an overnight TSB culture. Bacteria were then pelleted and resuspended in 500 μl of 50 mM Tris-HCl buffer (pH 8.3) that contained 50 mM disodium EDTA. Lysis of cells was conducted using 15 U of lysostaphin (Sigma, St. Louis, Mo.) and incubating at 37°C for 30 min. Lysis was completed by adding 1 ml of lysis buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 1% Triton X-100, 0.45% Igepal CA-630, 0.45% Tween 20, and 0.6 μg of proteinase K and incubating for 1 h at 56°C. Proteinase K was inactivated by heating at 95°C for 10 min.

***agr* genotyping.** In order to amplify the variable region of the *agr* gene in the *S. aureus* strains, nested primers were designed using *S. aureus* sequences available from GenBank and the DNASTAR (Madison, Wis.) software program. The sequences of the outer primers were 5′-ACCAGTTTGCCACGTATCTCA-3′ and 5′-AACCACGACCTTCACCTTTAGTAG-3′. Amplification was conducted in a total volume of 40 μl consisting of 10 μl of cell lysate, 4 μl of 10× buffer, 0.4 μl of deoxyribonucleoside triphosphate (dNTP) (25 mM [each] dATP, dCTP, dGTP, and dTTP), a 2 μM concentration of each primer, 2.4 μl of $MgCl_2$ (25 mM), 2.5 U of *Taq* DNA polymerase, and 8 μl of nuclease-free water. Reactions were cycled as follows: 95°C for 30 s, 64°C for 60 s, and 72°C for 120 s for 34 cycles.

For the nested primer amplification, 1 μl of the first PCR mixture was added to 39 μl of PCR mixture containing 2 μM concentrations of the second set of primers: 5′-TGCCACGTATCTTCAAA-3′ and 5′-ATAATCATGACGGAACT T-3′. The nested PCR amplification conditions were the same as above with an annealing temperature of 54 instead of 64°C.

Ten microliters of the second PCR mixture was digested at 37°C for 1 h with 2 U of the restriction endonuclease *Alu*I (Promega, Madison, Wis.) according to the manufacturer's instruction. The digested DNA fragments were separated in a 3% agarose gel (Sigma) and visualized in the presence of ethidium bromide under UV light.

Coagulase genotyping. The coagulase genotyping was performed by a previously described method (1). Ten microliters of DNA lysates was added to a mixture containing a 1 μM concentration of each primer (COAG1, 5′-ATACT CAACCGACGACACCG-3′, and COAG4, 5′-GATTTTGGATGAAGCGGAT T-3′), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 1% Triton X-100, a 200 μM concentration of each dNTP, and 1 U of *Taq* polymerase to a final reaction volume of 40 μl. Each sample was subjected to 40 PCR cycles, consisting of 30 s at 95°C, 2 min at 55°C, and 2 min at 72°C. For the nested-PCR amplification, 1 μl of the first PCR mixture was added to 39 μl of PCR mixture containing a 1 μM concentration of the second set of primers (COAG2, 5′-ACCACAAGGTACT GAATCAACG-3′, and COAG3, 5′-TGCTTTCGATTGTTCGATGC-3′). The nested-PCR amplification was performed using the same conditions as the first PCR. Ten microliters of the second PCR mixture was digested at 37°C for 1 h with 2 U of the restriction endonuclease *Alu*I according to the manufacturer's instruction. The digested DNA fragments were separated in 4% NuSieve GTG agarose gel (FMC BioProducts, Rockland, Maine) and detected in the presence of ethidium bromide under UV illumination.

Detection of enterotoxin genes by multiplex PCR. Enterotoxin typing was conducted according to the following methodology. In brief, staphylococcal genomic DNA was extracted from lysostaphin-treated cells and processed as described previously (20). The DNA was extracted with phenol-chloroform (1:1, vol/vol) and chloroform and then precipitated with ethanol according to standard techniques (28). Specific primers for staphylococcal enterotoxin A (SEA), SEE, and toxic shock syndrome toxin 1 (TSST-1) were synthesized with a DNA synthesizer (Expedite 8905; Perseptive Co.) as described previously by Johnson et al. (17, 18, 28). The oligonucleotide sequence of each primer is shown in Table 1. The PCR was performed under the following parameters: the reaction mixture consisted of 2.5 μl of 10× reaction buffer without $MgCl_2$ (Promega Corp.); 400 M dNTP; 3 mM $MgCl_2$; 7.5% dimethyl sulfoxide; 50 pmol of primers for *sea*, *seb*, *sec*, *sed*, and *see*; 100 pmol of primers for *tst*; and 100 ng of template DNA; and brought up to a 25-μl final volume with distilled water. Reactions were hot started for 5 min at 95°C and placed on ice, and 1 U of *Taq* polymerase (Promega Corp.) was added. Each sample was subjected to 30 PCR cycles, consisting of 95°C for 1 min, 2 min at 56°C (for the combination of primer sets for SEA, SEC, and SED) or 50°C (for SEB, SEE, and TSST-1), and 1 min at 72°C. PCR products were separated on a 1.5% agarose gel and visualized under UV illumination.

TABLE 1. Nucleotide sequences of primers used in multiplex PCR

Gene	Primer	5′-3′ sequence	Location of gene	Product size (bp)
sea	SEA-1	TTGGAAACGGTTAAAACGAA	490–509	121
	SEA-2	GAACCTTCCCATCAAAAACA	591–610	
seb	SEB-1	TCGCATCAAACTGACAAAGG	634–653	477
	SEB-2	GCAGGTACTCTATAAGTGCC	1091–1110	
SEC	SEC-1	GACATAAAAGCTAGGAATTT	676–695	257
	SEC-2	AAATCGGATTAACATTATCC	913–932	
sed	SED-1	TAGATAAAGTTAAAACAAGC	354–373	318
	SED-2	TAACTTACCGTGGACCTTC	652–671	
see	SEE-1	TAGATAAAGTTAAAACAAGC	491–510	169
	SEE-2	TAACTTACCGTGGACCCTTC	640–659	
tst	TST-1	ATGGCTATATACATTCAATT	251–270	350
	TST-2	TTTCCAATAACCACCCGTTT	581–600	

Statistical analysis. Discriminatory powers of both coagulase and *agr* genotyping were evaluated as described by Hunter (14). Concordance analysis (10) of *agr* genotype, coagulase genotype, and enterotoxin production capabilities was conducted by using the Minitab (State College, Pa.) statistical program to determine matches and mismatches among isolates within the same and different groups. Pairwise comparisons (number of combinations) were determined with the following formula: $P_{k,n}/k! = n!/k!(n - k)!$, where n = number of samples and k = number of combinations chosen. The G test of independence using Yate's correction for continuity (31) was used to evaluate statistical significance.

Bovine blood neutrophil bactericidal assay. The functional capabilities of bovine neutrophils are a major factor which determines the establishment of new intramammary infections. For this reason, a series of in vitro assays were conducted to assess the relative abilities of certain strains to resist this important host defense mechanism. Bovine neutrophils were isolated from four lactating Holstein cows free of intramammary infection as determined by microbiological analyses of milk samples. Neutrophils were isolated as previously described (2). For the purpose of opsonization, bovine antiserum was collected from cows diagnosed with *S. aureus* mastitis.

Ten *S. aureus* isolates were selected from both predominant (n = 5) and rare (n = 5) genotypes to evaluate differences in capabilities of prevalence groups to evade neutrophil killing. Additionally, eight *S. aureus* isolates were selected from predominant genotypes with enterotoxin genes (n = 4) and without enterotoxin genes (n = 4) to evaluate the effect of enterotoxin genes on neutrophil killing capabilities. Bacteria were prepared by initial culturing in 30 ml of assay medium (RPMI–5% fetal bovine serum–1% L-glutamine) at 37°C for 6 to 12 h. After incubation bacterial concentration was determined via serial dilutions. Bacterial inocula were stored at 4°C, while concentrations were determined and final concentrations were adjusted to 10^7 CFU/ml in assay medium. The resistance of bacteria to bovine neutrophil bactericidal activities was evaluated by the bactericidal assay as previously described (2). In brief, bacteria were opsonized in 6.25% bovine antiserum for 30 min at 37°C. In a 96-well plate, 100 μl of bacteria (10^7 CFU/ml) was combined with 100 μl of neutrophils (10^8/ml) and incubated

TABLE 2. *agr* genotype profiles of *S. aureus* strains ($n = 248$)

Type	*agr* restriction pattern subgroups[a]	% Occurrence
1	8-480,385,310,257,168,156,104,75	47.8
2	4-557,486,181,144	17.7
3	4-750,480,385,75	9.6
4	6-452,373,188,144,104,75	9.6
5	3-680,557,144	4.4
6	5-680,557,486,181,144	1.6
7	3-750,385,66	1.2
8	5-500,257,168,156,75	1.2
9	6-620,480,385,188,104,75	0.8
10	4-848,257,140,70	0.8
11	4-480,257,168,156	0.8
12	6-668,405,362,150,104,75	0.8
13	3-557,486,144	0.8
14	6-747,449,395,333,257,75	0.4
15	3-570,480,257	0.4
16	3-330,255,168	0.4
17	2-643,557	0.4
18	5-680,385,181,114,75	0.4
19	7-557,480,385,181,168,104,75	0.4
20	6-680,480,410,287,104,75	0.4

[a] First digit indicates number of bands; subsequent values indicate the molecular weight (in thousands) of each band.

at 37°C for 1 h. Following incubation, neutrophils were lysed with the addition of 0.2% Saponin (Sigma) followed by the addition of MTT (1 mg/ml; Sigma). Upon color development, the addition of extraction buffer lysed bacterial cells. Plates were read at a wavelength of 595 nm on a microplate reader (Bio-Rad, Hercules, Calif.).

Additional assays were conducted to determine if SEA, the enterotoxin gene type observed most frequently in field isolates, could affect neutrophil bactericidal activity. For experiments involving SEA (Sigma), neutrophils were preincubated with various concentrations of enterotoxin A for 15 min at 37°C in order to determine the direct effect of this enterotoxin on neutrophil killing capabilities. The dessicated SEA contained 10% protein and 90% sodium phosphate buffer by weight; therefore, neutrophils were preincubated with 4 mM sodium phosphate, the highest concentration found when 20 μg SEA was tested, to serve as control. Bacterial strain Newbould 305 was used to determine the effect of SEA on neutrophil bactericidal capability. Bacteria were inoculated as described above and washed three times in phosphate-buffered saline to remove any secreted enterotoxin produced during growth. Bacteria were then resuspended in assay medium and counted as described above. Student's *t* test was used to compare percentages of bacteria killed for strains within each prevalence or treatment group.

Detection of enterotoxin protein. *S. aureus* strains used in the neutrophil bactericidal assays for toxin evaluation were further studied to determine protein production of enterotoxins under assay conditions. Bacteria were prepared by initial culturing in 30 ml of assay medium (RPMI–5% fetal bovine serum–1% L-glutamine) at 37°C for 6 to 12 h. After incubation, bacterial cultures were centrifuged at 3,500 × *g* for 5 min at 15°C. Supernatants were then filter sterilized and used in an enzyme immunoassay for the detection of enterotoxins A, B, C, D, and E (RIDASCREEN Set A,B,C,D,E; r-biopharm, Darmstadt, Germany).

RESULTS

***agr* genotyping.** Available *agr* sequences were analyzed in order to determine which region of the gene had the largest diversity to ensure that a variety of restriction fragment length polymorphism profiles would be attained (Table 2). DNA sequence analysis of several *S. aureus* strains showed highly conserved regions at the 5′ end of the *agrB* gene and the 3′ end of the *agrC* gene (GenBank sequence accession numbers AF001782, AF001783, U85095, and X52543). These conserved sequences were used to design primers to amplify *agr* gene fragments displaying areas of high divergence found within the stable regions. Specificity of the designed primers was confirmed using *Escherichia coli* and an *agr S. aureus* mutant RN6391 (Fig. 1). Sequencing of PCR products from *S. aureus* ATCC 27217 and *S. aureus* RN6390 confirmed that the PCR product was the targeted region of the *agr* locus an expected product of approximately 1,386 bp.

S. aureus strains collected from clinical bovine milk samples from Czech Republic, France, Korea, and several states in the United States were genotyped by *agr* gene polymorphism. Twenty genotypes have been identified, and an example of an agarose gel electrophoresis is shown in Fig. 2. Genotype profiles were designated by the total number of major bands followed by the estimated molecular weight (in thousands) of each band. The most prevalent type was found to have eight major bands with molecular weights of 480, 385, 310, 257, 168, 156, 104, and 75 and was therefore designated 8:480, 385, 310, 257, 168, 156, 104, 75. The frequencies of all the identified *agr* genotypes within the tested samples are shown in Table 2. From a total of 255 strains tested, seven samples were nontypeable using *agr* genotyping methods. *agr* genotyping methodology had a discriminatory power (*D*) of 0.7217.

Coagulase genotyping. The 255 available isolates were subdivided into 40 different coagulase types according to previously designated typing patterns (1). There were four samples that were nontypeable using the coagulase genotyping method. Coagulase typing had a very high *D* of 0.938. As shown previously, only a few genotypes predominate in each country (32). However, comparisons among countries indicated that predominant types could be distributed in different geographical locations (32).

Detection of enterotoxin genes by multiplex PCR. The presence of enterotoxin genes was assessed in 211 isolates randomly chosen from the available 255 isolates from various locations. Of the isolates tested, 118 isolates tested negative for enterotoxin genes (56%), and 93 isolates were positive for enterotoxin genes (44%). Of the isolates positive for enterotoxin genes 58 were positive for SEA (62.4%) only, 3 were positive for SEB (3.2%) only, 5 were positive for SEC (5.4%) only, 3 were positive for SED (3.2%) only, and 4 were positive for TSST (4.3%) only. In addition, 20 of the isolates tested positive for more than one enterotoxin gene: 4 isolates were positive for both SEA and SEC (4.3%); 2 isolates were positive for SEA, SEC, and TSST (2.15%); 2 isolates were positive for SEA and TSST (2.15%); 3 isolates tested positive for SEC, SED, and TSST (3.2%); and 9 isolates possessed SEC and TSST (9.7%) genes. Selected strains of *S. aureus* used in the

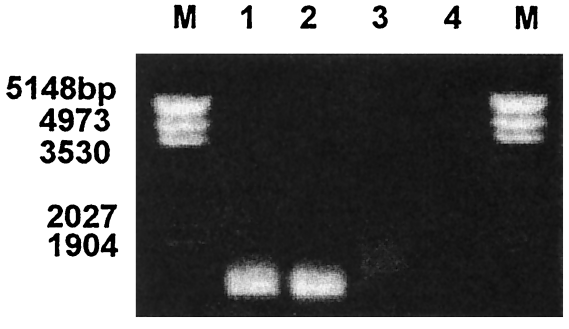

FIG. 1. Nested PCR products of *S. aureus* ATCC 27217 (lane 1), RN6390 (lane 2), *agr* mutant RN6391 (lane 4), and *E. coli* (lane 3).

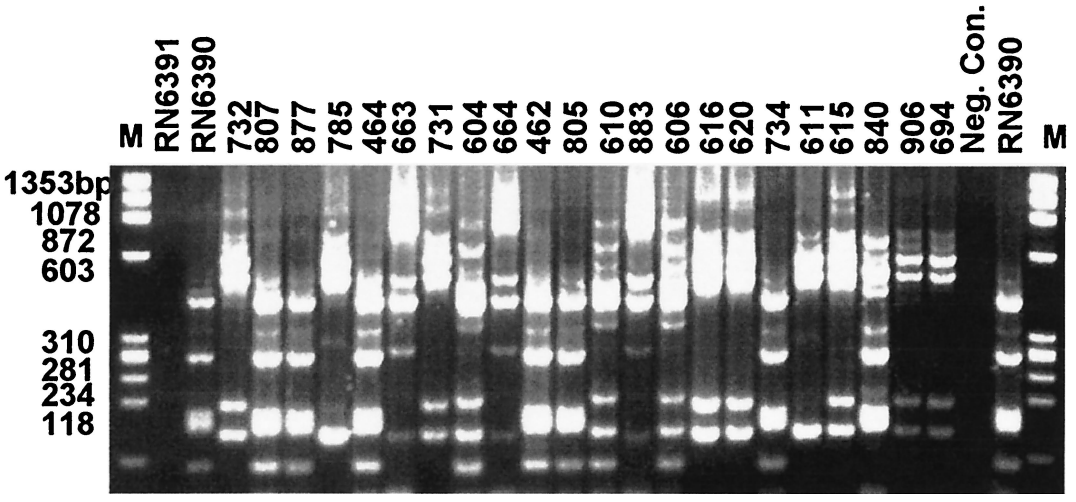

FIG. 2. Example of restriction fragment profiles of the *agr* gene for 26 *S. aureus* strains.

bactericidal assays also were evaluated for expression of enterotoxin genes. Strains with enterotoxin genes were found to produce the respective enterotoxin protein as evaluated through an enzyme immunoassay. Additionally, strains that tested negative for enterotoxin genes did not test positive for any enterotoxin protein production under assay conditions (data not shown).

Concordance analyses of typing techniques. All possible pairs of *agr* genotyping and coagulase genotyping were compared among the 248 samples that were typeable using the two techniques. Pairs of isolates were classified by whether they possessed the same or different *agr* genotypes and whether they matched or mismatched in coagulase genotypes (Table 3). A total of 30,628 pairwise comparisons for the 248 isolates were possible. For the isolates with the same *agr* type, 4% had matching and 23% had mismatching coagulase genotypes. For the isolates with different *agr* types, 2% had matching and 71% had mismatching coagulase genotypes. The overall concordance percentage for coagulase and *agr* genotypes was 75.2% (simple matching coefficient [S] = 0.752) and had a significance of $P < 0.001$ (G test of independence, $G = 1621.4$; df = 1).

Concordance analysis of genotyping methods and enterotoxin typing. Of the 211 isolates tested for enterotoxin type, 6 isolates had unidentifiable *agr* genotypes and 3 isolates had unidentifiable coagulase types. Pairs of isolates were identified by whether they had the same or different enterotoxin type and whether they matched or mismatched in either *agr* genotype or coagulase genotype (Table 4). Of the total of 21,910 possible pairwise comparisons between enterotoxin type and *agr* genotype (205 isolates), 10% of the same enterotoxin type had matching *agr* genotypes and 29% had mismatching *agr* genotypes. For the isolates with different enterotoxin types, 17% had matching and 44% had mismatching *agr* genotypes. The overall concordance of enterotoxin type with *agr* genotype was 54.3% (simple matching coefficient, $S = 0.543$) and had a significance of $P < 0.001$ (G test of independence, $G = 14.2$; df = 1).

In the total of 21,528 possible pairwise comparisons between enterotoxin type and coagulase genotype (208 isolates), 3% of same enterotoxin type had a matching coagulase genotype and 36% had a mismatching coagulase genotypes. For the isolates with different enterotoxin types, 3% had matching and 58% had mismatching coagulase genotypes. The overall concordance of enterotoxin type with coagulase type was 61.2% (simple matching coefficient, $S = 0.612$) and had a significance of $P < 0.001$ (G test of independence, $G = 64.4$; df = 1).

Bovine blood neutrophil bactericidal assay. The results of bactericidal assays are shown in Fig. 3 to 5. The mean percentage of killing was 41% (standard error [SE] = 1.4) for the five high prevalence *S. aureus* strains and 59% (SE = 0.9) for the five low-prevalence strains evaluated. There was a significant

TABLE 3. Concordance analysis of *agr* gene type and coagulase gene type[a]

agr type	No. of isolate pairs (proportion of total comparisons) with coagulase type	
	Match	Mismatch
Same	1,335 (0.04)	6,998 (0.23)
Different	575 (0.02)	21,720 (0.71)

[a] Concordance equals the sum of the same coagulase-match and different coagulase-mismatch entries, expressed as a percentage of the total 30,628 pairwise comparisons. For data presented here, the concordance is 75.2%.

TABLE 4. Concordance analysis of enterotoxin type with *agr* genotype and coagulase genotype[a]

Enterotoxin	No. of isolate pairs (proportion of total comparison) with:			
	agr genotype		Coagulase genotype	
	Match	Mismatch	Match	Mismatch
Same	2,012 (0.10)	6,038 (0.29)	636 (0.03)	7,699 (0.36)
Different	3,518 (0.17)	9,342 (0.44)	659 (0.03)	12,543 (0.58)

[a] Concordance equals the sum of the same enterotoxin-match and different enterotoxin-mismatch entries, expressed as a percentage of the total pairwise comparisons, i.e., 21,910 for *agr* genotype and 21,528 for coagulase genotype. Thus, the concordance for *agr* genotype is 54.3%, and that for coagulase genotype is 61.2%.

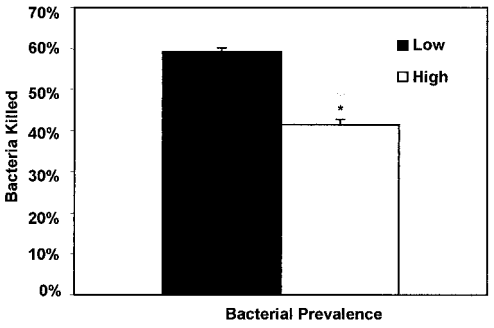

FIG. 3. Bovine blood neutrophil bactericidal activity against either high- or low-prevalence *S. aureus* strains (*, $P < 0.005$). Error bars, SE.

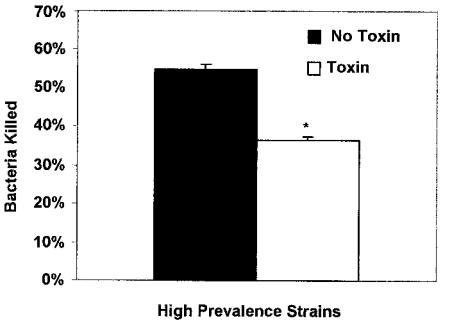

FIG. 4. Bovine blood neutrophil bactericidal activity against high-prevalence *S. aureus* strain with or without enterotoxin genes. *, $P = 0.005$; error bars, SE.

difference in bactericidal effects ($P = 0.004$). Additionally, the mean percentage of killing was 35% (SE = 1.6) for four high-prevalence strains with enterotoxin genes and 54% (SE = 1.2) for strains without enterotoxin genes. A significant difference was found in bactericidal effects ($P = 0.005$) between those strains with and without enterotoxin genes. Strains with enterotoxin genes were found to produce the respective enterotoxin protein as evaluated through an enzyme immunoassay (data not shown). Additionally, strains that tested negative for enterotoxin genes did not test positive for any enterotoxin protein production under assay conditions. Addition of exogenous SEA to the bactericidal assay resulted in a significant decrease in neutrophil killing capabilities ($P < 0.05$) at concentrations of 5, 15, and 20 μg.

DISCUSSION

Previous work in our laboratory has used the hypervariable region of the coagulase gene to type *S. aureus* strains (32). The coagulase gene was chosen due to its ubiquitous presence among *S. aureus* strains, and this gene proved to create a very powerful typing method. In this study, the *agr* gene also was used to further type strains and provided a means of grouping *S. aureus* isolates based on a factor that controls virulence-related gene production. The *agr* gene was used successfully to subtype *S. aureus* strains, and when the information attained from genotyping of the coagulase gene and the *agr* gene was combined, the discriminatory power of each method was greatly empowered. The ability to group pathogens based on field prevalence may provide important information pertaining to the coevolution of hosts and pathogens which will influence the genetic diversity of disease-causing microorganisms (13, 22). Selection within the pathogen population will favor mechanisms to avoid host defense and to colonize the host. The pathogens that are most efficient at avoiding the host's defense mechanisms will be the most prevalent type found in the microenvironment of interest. The argument for this phenomenon is supported by the findings in this study in which the abilities of neutrophils to kill different *S. aureus* genotypes varied with respect to their prevalence in cases of mastitis. We discovered that the most common genotype of *S. aureus* also was the type against which the neutrophils, and thereby the host's initial defense mechanism, were least efficient. In contrast, the neutrophils were highly efficient against the rarely found genotypes. These results are consistent with our previous reports (2, 32) and suggest that types found in high prevalence have unique characteristics, which in contrast to the rare types, endow them with the superior ability to suppress or resist killing by neutrophils. In the past, in vitro studies of host-pathogen interactions have focused on the use of few selective bacterial strains studied routinely in a laboratory environment (3, 4, 12). However, statistical analysis of a large database of pathogenic strains may provide critical information concerning key virulence-related genes in a prevalent group of disease-causing bacteria that can be evaluated relative to the interaction between pathogen and host in a specific disease model.

Bacterium-host interactions depend on several factors, including the efficiency of the host's defense, growth rate of the bacteria, and production of virulence factors. The neutrophils used in the assays were obtained from the same group of animals, so differences in killing ability cannot be attributed to variations in host defense. In addition, no differences in the growth potential were observed among the strains used in the bactericidal assays. One possible explanation for the variation in killing efficiency may be a consequence of the expression of certain bacterially produced factors. *S. aureus* has the capacity to synthesize a repertoire of known virulence factors associated with mastitis, including capsular polysaccharide, cell-surface associated proteins, and several hemolytic toxins (33). However, the potential role of enterotoxins in the pathogenesis of *S. aureus* mastitis is uncertain and has been the topic of several

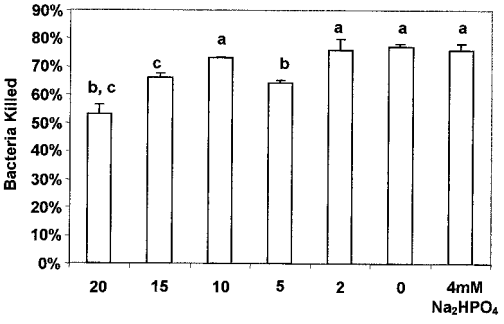

FIG. 5. Bovine blood neutrophil bactericidal activity against *S. aureus* strain Newbould 305 in the presence of enterotoxin A. Bars labeled with different letters indicate that values differ significantly ($P < 0.05$). Error bars, SE.

conflicting studies (9, 19). In this study, the use of multiplex PCR technology of the enterotoxin gene was conducted to evaluate the significance of this group of potential virulence factors associated with mastitis-causing *S. aureus* strains. Use of this technique to evaluate enterotoxin genes eliminated the variations previously observed for enterotoxin detection as a result of various growth conditions influencing the cell density sensing system that controls virulence factor production in *S. aureus* (16). We found that 44% of *S. aureus* isolates tested contained enterotoxin genes, and these values are consistent with previous published findings using similar methodologies (20). The significant relationship between enterotoxin types and *agr* genotypes reported here is supported by previous work in which differences were observed in toxin production due to mutations in the *agr* gene (11, 25).

It was interesting to further delineate if the ability of prevalent *S. aureus* strains to evade host defense mechanisms was related, at least in part, to enterotoxin production capabilities. We showed that strains with enterotoxin genes were significantly ($P = 0.005$) better at evading this nonspecific cellular defense mechanism of the host compared to strains without these genes. *S. aureus* enterotoxins have been shown to activate T-cell subsets (34) as well as provide protection against neutrophil apoptosis (23). However, very limited information is available as to the effect of enterotoxins on neutrophil bactericidal activities. In order to establish a direct link between status of the *agr* gene, enterotoxin genes, and neutrophil killing capabilities, specific *S. aureus* enterotoxins were studied for the ability to alter bactericidal activities of neutrophils. We assessed the direct effects of SEA on neutrophil bactericidal capabilities since this enterotoxin gene was most commonly observed among the most prevalent *agr* genotypes. The addition of exogenous SEA was shown to significantly reduce the killing abilities of neutrophils under our assay conditions. Previously, work by Berger et al. (8) observed no effect on SEA on neutrophil bactericidal activity. However, the preincubation of neutrophils with enterotoxin prior to evaluation of killing abilities, as carried out in the present study, may be the deciding factor in the varying results observed. Though there are several putative virulence factors that can account for the effect on neutrophil killing ability observed in Fig. 3, singling out SEA, the most-common enterotoxin gene possessed by the isolates in this study provides a direct correlation between variations in the *agr* gene sequences, enterotoxin production, and ability to evade neutrophil killing.

The epidemiological study of a large database of disease-causing bacteria can be used to identify uniquely expressed virulence factors that are associated with the ability of pathogens to evade important host defense mechanisms. Using this approach, we showed for the first time that staphylococcus enterotoxins can contribute to the pathogenesis of *S. aureus* mastitis as suggested by the *agr* genotype field prevalence data. We also showed that one possible mechanism by which SEA may affect virulence is through its ability to directly hinder neutrophil bactericidal activities. A better understanding of important host-pathogen interactions that contribute to field prevalence of a specific disease may provide greater insight into the development of effective intervention strategies.

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