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Staphylococcus aureus Isolates from Patients with Kawasaki Disease Express High Levels of Protein A

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Kawasaki disease (KD) is an acute vasculitis of young children that can be complicated by coronary artery abnormalities. Recent findings suggest that a superantigen(s) may play an important role in stimulating the immune activation associated with the disease, although the origin of this superantigen(s) is unclear. *Staphylococcus aureus*, isolated from the rectum or pharynx of patients with KD, secretes toxic shock syndrome toxin 1 (TSST-1). The KD isolates express low levels of other exoproteins compared to isolates from patients with toxic shock syndrome (TSS). Thus, it was previously suggested that the KD isolates may be defective in the global regulatory locus *agr* (for accessory gene regulator), which positively regulates these factors (D. Y. M. Leung et al., Lancet 342:1385–1388, 1993). Here we describe another characteristic of KD isolates. When considered collectively, the KD isolates were found to express higher levels of staphylococcal protein A than the TSS isolates, another characteristic of an *agr*-defective phenotype. This correlated with a higher level of *spa* mRNA in these isolates. In contrast, the KD and TSS isolates expressed comparable levels of TSST-1, consistent with previous findings (D. Y. M. Leung et al., Lancet 342:1385–1388, 1993). Analysis of RNAIII transcript levels and nucleotide sequence analysis of the RNAIII-coding region suggested that the KD isolates are not defective in RNAIII, the effector molecule of the *agr* regulatory system. However, induction of RNAIII transcription in the KD isolates did not result in a dramatic decrease in the amount of *spa* mRNA, as has been reported for other strains (F. Vandenesch, J. Kornblum, and R. P. Novick, J. Bacteriol. 173:6313–6320, 1991).

Kawasaki disease (KD) is an acute illness of early childhood characterized by fever, induration and erythema of the hands and feet, inflammation of the mucous membranes, polymorphous skin rash, and cervical lymphadenopathy (23). As a complication of this multisystem vasculitis, coronary artery aneurysms or ectasias occur in approximately 25% of untreated patients (22). In the United States and Japan, KD is currently the leading cause of acquired heart disease in children. A number of observations suggest that immune mechanisms play an important role in the pathogenesis of the disease. In particular, acute KD is characterized by significant immune activation. Increased numbers of circulating activated T cells, B cells, and macrophages/monocytes lead to high levels of circulating cytokines and to the production of cytotoxic antibodies directed against vascular endothelial cell antigens (14, 30–32). These immunologic features, particularly the activation of T cells and macrophages, are characteristic of diseases which are caused by microbial superantigens (25). Indeed, the clinical symptoms and epidemiology of KD are highly suggestive of an infectious disease, overlapping with staphylococcal and streptococcal scarlet fevers and toxic shock syndromes (TSSs).

Recently, it was suggested that staphylococcal toxic shock syndrome toxin 1 (TSST-1) and streptococcal pyrogenic exotoxins might contribute to KD (34). In a blinded, controlled study, 13 of 16 KD patients were found to be infected with TSST-1-producing ($n = 11$) or streptococcal pyrogenic exotoxin C-producing ($n = 2$) bacteria, compared with only 1 of 15 febrile controls. We proposed that TSST-1-producing *Staphylococcus aureus* and streptococcal pyrogenic exotoxin C-producing streptococci could account for the selective expansion of Vβ2-expressing T cells in the blood and tissues of patients with acute KD, which has now been reported by three independent groups (1, 2, 11, 33, 50). In a follow-up multicenter study, it was found that TSST-1-producing *S. aureus* could be isolated from 31 of 32 patients with acute KD (36).

Staphylococcal protein A (SpA) is produced by over 95% of *S. aureus* strains and has the ability to bind to the Fc region of immunoglobulin G (IgG) in most mammalian species. This protein has been shown to inhibit opsonization and phagocytosis of staphylococci in vitro, a factor that may contribute to the virulence of this organism in vivo (15, 42, 43). SpA also exhibits diverse immunobiological properties, including an ability to activate B cells (B-cell superantigen) and complement (17, 26–28, 48). Interestingly, these laboratory parameters of immune activation have been reported during acute KD (24, 29). Thus, together with T-cell superantigens, such as TSST-1, SpA could account for at least some of the immune activation associated with this disease.

It was recently observed that *S. aureus* isolated from KD patients produced less lipase, hemolysin, and protease than isolates from TSS and other skin diseases. However, the KD isolates did not produce particularly low levels of TSST-1 (34). The expression of these exoproteins is controlled by global regulatory loci, including *agr* (for accessory gene regulator) (38, 44). This locus consists of two divergent transcripts, RNAII and RNAIII, driven by two distinct promoters, P2 and P3, respectively (40). RNAIII is the effector molecule of this

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regulatory system. During the postexponential and stationary phases of growth, this molecule upregulates the expression of various exoproteins and downregulates the expression of factors such as SpA, the fibronectin-binding proteins, and coagulase (20, 41). In bona fide *agr*-defective mutants, there is a lack of RNAIII expression, low expression levels of the positively regulated extracellular factors such as hemolysins, proteases, and TSST-1, and high expression levels of the negatively regulated factors, such as SpA. It has thus been speculated that the KD isolates may represent a new clone of TSST-1-secreting *S. aureus* which has a partially defective *agr* locus, or these isolates may be bona fide *agr*-defective mutants which have reverted in their ability to express TSST-1 (34).

In this study, we further characterized the KD isolates by examining the level of SpA expressed by these isolates compared to TSS isolates. We report that, when considered as a group, the KD isolates express significantly higher levels of cell wall-associated and extracellular SpA than TSS isolates, another characteristic of an *agr*-defective phenotype. However, consistent with previous observations, no difference was seen in the level of TSST-1 expressed by the two groups of isolates. We investigated the possibility that the KD isolates may be partially defective in their *agr* loci by analyzing *spa* and RNAIII transcription levels and by nucleotide sequence analysis of the RNAIII-coding region.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. aureus* strains Cowan I and Wood 46 were obtained from the American Type Culture Collection (Rockville, Md.). *S. aureus* strains 8325-4, RN4282, and RN4256 (RN4282 *agrA*::Tn*551*) were kindly provided by R. P. Novick (Public Health Research Institute, New York, N.Y.). TSST-1-expressing *S. aureus* isolates were obtained from either pharyngeal or rectal swabs of 21 patients with acute KD. Each of these patients fulfilled the diagnostic guidelines of the American Heart Association (3). TSST-1-expressing vaginal isolates of *S. aureus* were obtained from 12 patients with menstrual TSS. *S. aureus* strains were grown in tryptic soy broth (TSB). *Escherichia coli* JM101 (51) was used for plasmid DNA transformations and was grown in Luria-Bertani broth, containing 100 μg of ampicillin per ml when appropriate.

Analysis of cell wall-associated SpA expression by flow cytometry. Overnight cultures were diluted 1:50 in 5 ml of fresh TSB and were grown with shaking at 37°C to early and late exponential phases (Fig. 1A, t_1 and t_2, respectively). All the isolates had similar growth rates and equivalent optical density at 600 nm (OD_{600}) values at each 1-h time point. Cells were pelleted at 2,500 × *g* for 10 min at 4°C and then resuspended and blocked in staining solution (5% fetal calf serum, 0.1% intravenous immunoglobulin, 0.02% sodium azide in phosphate-buffered saline [PBS]) for 1 h at 4°C. Following the addition of 0.5 μg of biotinylated anti-SpA monoclonal antibody (MAb) (Sigma, St. Louis, Mo.), the cells were incubated for a further 30 min at 4°C. Cells were then washed twice in a wash solution (2% fetal calf serum, 0.02% sodium azide in PBS) and resuspended in staining solution. Phycoerythrin-conjugated streptavidin (Southern Biotechnology Associates, Inc., Birmingham, Ala.) was added, and the cells were incubated for 30 min at 4°C. Cells were then washed once in wash solution and twice in 0.02% sodium azide in PBS. Stained cells were fixed with 1% methanol-free formaldehyde in PBS (Polysciences, Inc., Warrington, Pa.) and analyzed with a Becton Dickinson FACScalibur, by using Cellquest software. Relative cell wall-associated SpA expression was measured by mean fluorescence intensity (MFI) with the anti-SpA MAb.

Quantification of extracellular SpA expression by ELISA. Extracellular SpA production was quantified by enzyme-linked immunosorbent assay (ELISA), as previously described (13). Overnight cultures were diluted 1:50 in 5 ml of fresh TSB and were grown with shaking at 37°C to early and late exponential phases (Fig. 1A, t_1 and t_2, respectively). All the isolates had similar growth rates and equivalent OD_{600} values at each time point. Cells were pelleted at 2,500 × *g* for 10 min at 4°C, and the supernatants were retained for analysis. Each well of a 96-well polystyrene plate (Corning, New York, N.Y.) was coated with 50 μl of a solution containing 10 μg of anti-SpA MAb (Sigma) per ml of 0.1 M $NaHCO_3$ buffer (pH 8.2) for 18 h at 4°C. The wells were washed twice with PBS (pH 7.2) containing 0.05% Tween 20 (PBS/T) and then blocked with 200 μl of 1% bovine serum albumin (BSA) in PBS/T per well for 2 h at room temperature. After washing twice with PBS/T, 100 μl of a 1:10 dilution (in PBS/T containing 1% BSA) of each supernatant was added to the wells and incubated for 18 h at 4°C. The wells were washed four times with PBS/T, 100 μl of biotinylated anti-SpA MAb (Sigma) in PBS/T was added to each well, and the plate was incubated for 45 min at room temperature. After washing six times with PBS/T, 100 μl of avidin-peroxidase conjugate (Sigma) in PBS/T-BSA was added to each well, and the plate was incubated for 30 min at room temperature. Wells were washed eight times with PBS/T, 100 μl of ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid] peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added to each well, and the plate was incubated at room temperature in the dark. After 9 min, the reaction was quenched by adding 100 μl of stop solution to each well. The OD_{405} was determined. A standard curve was prepared with known concentrations of a commercial preparation of SpA, purified from the cell wall of *S. aureus* Cowan I (Sigma).

Quantification of TSST-1 expression by ELISA. Overnight cultures were diluted 1:50 in 5 ml of fresh TSB and were grown with shaking at 37°C to late exponential phase (Fig. 1A, t_2). All the isolates had similar growth rates and equivalent OD_{600} values at each time point. Cells were pelleted at 2,500 × *g* for 10 min at 4°C. The supernatants were diluted 1:4 in PBS/T, normal rabbit serum was added to a final concentration of 1% (vol/vol), and the samples were incubated for 15 min at room temperature. Each well of a 96-well polystyrene plate (NUNC, Inc., Naperville, Ill.) was coated overnight with 100 μl of a solution containing 10 μg of anti-TSST-1 antibodies (Toxin Technology, Sarasota, Fla.) per ml of 0.1 M $NaHCO_3$ buffer (pH 8.2) at 37°C. The plate was then washed twice with PBS/T and blocked with PBS/T for 30 min at room temperature. After washing twice with PBS/T, 100 μl of each diluted supernatant sample was added to the wells, and the plate was incubated for 2 h at 37°C. The plate was washed three times with PBS/T and then incubated with horseradish peroxidase-conjugated anti-TSST-1 antibodies (Toxin Technology) for 1 h at 37°C. Wells were washed 10 times with PBS/T, 100 μl of ABTS peroxidase (Sigma) was added to each well, and the plate was incubated at room temperature. After 15 min, the reaction was quenched by adding 50 μl of stop solution to each well. OD_{405} was determined. A standard curve was prepared with known concentrations of purified TSST-1 (Toxin Technology).

Isolation of RNA and Northern hybridization analysis. Total cellular RNA was isolated from cultures grown to early and late exponential phases (Fig. 1A, t_1 and t_2, respectively) by using the RNeasy Mini kit (Qiagen Inc., Santa Clarita, Calif.). All the isolates had similar growth rates and equivalent OD_{600} values at each time point. Ten micrograms of each RNA sample (as determined by measuring the OD_{260}) was resolved by 1.2% agarose-formaldehyde gel electrophoresis. RNA Millenium Markers (Ambion Inc., Austin, Tex.) were used as size standards. RNA was transferred onto a Hybond N+ membrane (Amersham Life Science, Inc., Arlington Heights, Ill.) and was hybridized with radioactively labeled DNA probes at 42°C, as described by Sambrook et al. (45). Probes were random primer labeled with [α-^{32}P]dCTP to a specific activity of 1×10^8 to 5×10^8 cpm/μg by using the RadPrime DNA labeling system (Gibco BRL, Grand Island, N.Y.). Probe DNA for the *spa* and RNAIII transcripts was amplified by PCR from the chromosomes of strains Cowan I and 8325-4, respectively, as follows: *spa*, a 1.346-kb fragment (nucleotide [nt] 771 to 2117, according to Shuttleworth et al. [46]) by using the primers 5′-TTATATCTGGTGGCGTAACACC TGC-3′ and 5′-ATGCTTGAGCTTTGTTAGCATCTGC-3′, and RNAIII, a 945-bp fragment (nt 581 to 1526, according to Janzon et al. [21]) containing the entire RNAIII-coding sequence and the 5′ half of *agrB* by using the primers 5′-AAATACATAGCACTGAGTCCAAGG-3′ and 5′-TTTAATAAGTCGCA CAGGAATGGG-3′. After hybridization, membranes were washed twice in a solution containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 65°C and once in 0.1× SSC–0.1% SDS at 65°C, for 30 min each time. Membranes were exposed to Kodak X-Omat AR film at −80°C for various lengths of time.

Molecular cloning and sequencing. An 862-bp fragment containing the entire RNAIII-coding region and approximately 160 bp of upstream sequence (nt 314 to 1175, according to Janzon et al. [21]) was amplified by PCR from the chromosomes of strain 8325-4, five KD isolates (Yeh, KD1, KD6, KD-ATK, and Thomas), and two TSS isolates (MN8 and FRI 1169) by using *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and the primers 5′-ACCTTTTCCAAC ATTAGACTTATT-3′ and 5′-TTATTTCTCTTTTGAAGATACGTGG-3′. The products were cloned into the TA cloning vector pCR2.1 (Invitrogen, San Diego, Calif.). The constructs were purified with the QIAprep Spin Miniprep kit (Qiagen) and were sequenced in both directions with the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, Calif.), M13 reverse and T7 primers, and an automated DNA sequencer (model 377; Perkin-Elmer Applied Biosystems) at the University of Texas—Houston Medical School Molecular Genetics Core Facility. Nucleotide sequences were analyzed with SeqEd software (Perkin-Elmer Applied Biosystems).

RESULTS

Determination of cell wall-associated SpA expression levels. The levels of cell wall-associated SpA produced by early- and late-exponential-phase (Fig. 1A, t_1 and t_2, respectively) cultures of the KD and TSS isolates were compared by flow cytometry, by using a biotinylated anti-SpA MAb and phycoerythrin-conjugated streptavidin. The MFI observed gives a relative measure of the cell wall-associated SpA expressed by each isolate (Fig. 2). Strains Cowan I and Wood 46 were

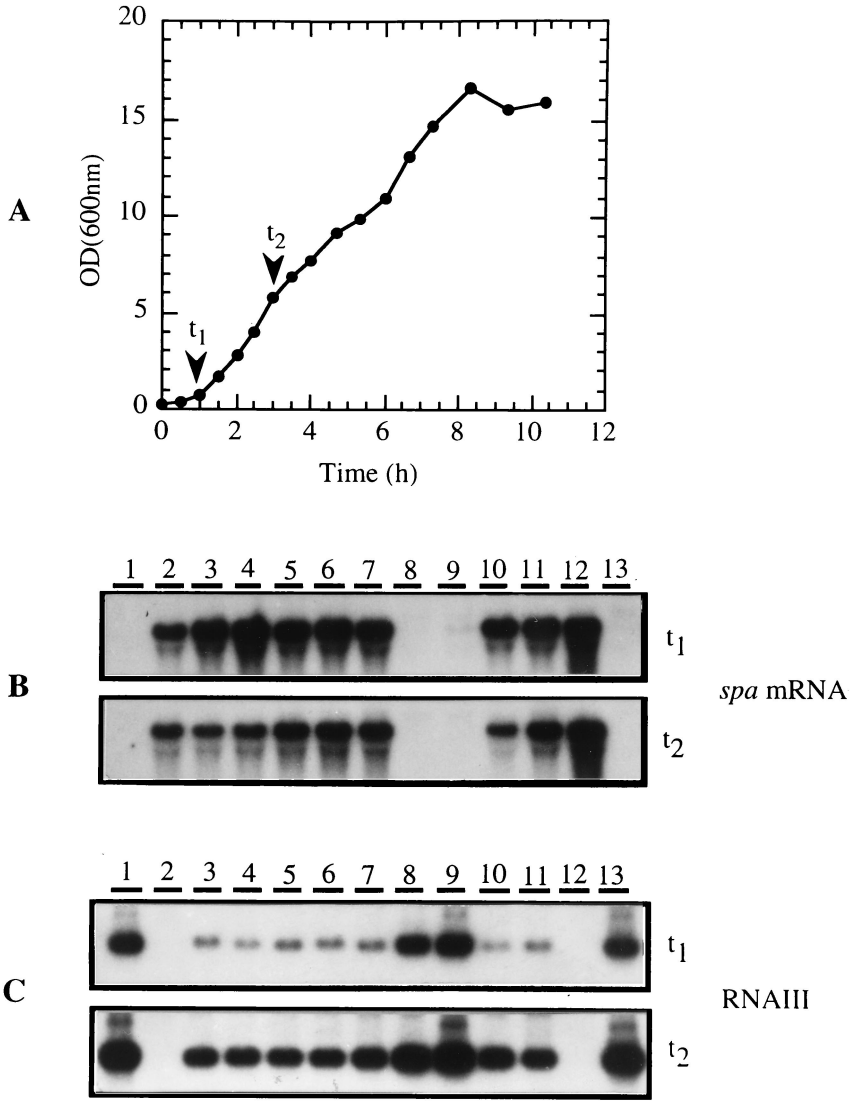

FIG. 1. (A) Growth curve of *S. aureus* Cowan I. Growth curves obtained for the KD and TSS isolates were very similar. Time points (t_1 and t_2 represent early and late exponential phases, respectively) at which samples were taken for isolation of total RNA for Northern hybridization analysis are indicated by the arrows. Ten micrograms of total RNA was loaded in each well and probed for the (B) *spa* and (C) RNAIII transcripts, as described in the text. Samples were loaded as follows: lane 1, strain RN4282; lane 2, strain RN4256 (strain RN4282 *agrA*::Tn*551*); lanes 3 to 7, KD isolates (strains Yeh, KD1, KD6, KD-ATK, and Thomas, respectively); lanes 8 to 11, TSS isolates (strains MN8, FRI 1169, L. Park TN 1986, and K. Johnson MN 1985, respectively); lane 12, strain Cowan I; and lane 13, strain Wood 46. This analysis was performed in duplicate, with similar results.

included in this study as examples of strains which express high and very low levels of SpA, respectively. Strain RN4256 was also included as an example of an *agr* mutant that lacks RNAIII, a negative regulator of *spa* gene transcription. As anticipated, strains Cowan I and RN4256 produced significantly higher levels of cell wall-associated SpA than strain Wood 46 at both growth phases analyzed. When compared collectively, the KD isolates ($n = 14$) expressed significantly higher levels of cell wall-associated SpA than the TSS isolates ($n = 11$) at the early exponential growth phase (MFI of 950.04 ± 349.38 versus 95.98 ± 58.79; $P < 0.05$, Student's t test). The same result was observed for late-exponential-phase cultures of the KD and TSS isolates (MFI of 1,597.07 ± 368.70 versus 228.99 ± 134.90; $P < 0.05$, Student's t test).

Production of extracellular SpA. The levels of extracellular SpA secreted by early- and late-exponential-phase cultures (Fig. 1A, t_1 and t_2, respectively) of the KD and TSS isolates were determined by ELISA (Fig. 3). Again, strains Cowan I, Wood 46, and RN4256 were included in the study as controls. When compared collectively, the KD isolates ($n = 21$) were found to secrete significantly higher levels of SpA than the TSS isolates ($n = 12$) at both growth phases analyzed (0.54 ± 0.10 versus 0.28 ± 0.22 μg/ml for early-exponential-phase cultures and 1.22 ± 0.42 versus 0.51 ± 0.35 μg/ml for late-exponential-phase cultures; $P < 0.05$, Student's t test). However, when considered individually, a few TSS isolates were observed to express high levels of extracellular SpA comparable to the KD isolates.

Production of TSST-1. The amounts of TSST-1 secreted by late-exponential-phase cultures (Fig. 1A, t_2) of the KD ($n = 19$) and TSS ($n = 12$) isolates were measured by ELISA (Fig. 4). The level of TSST-1 secretion was highly variable among

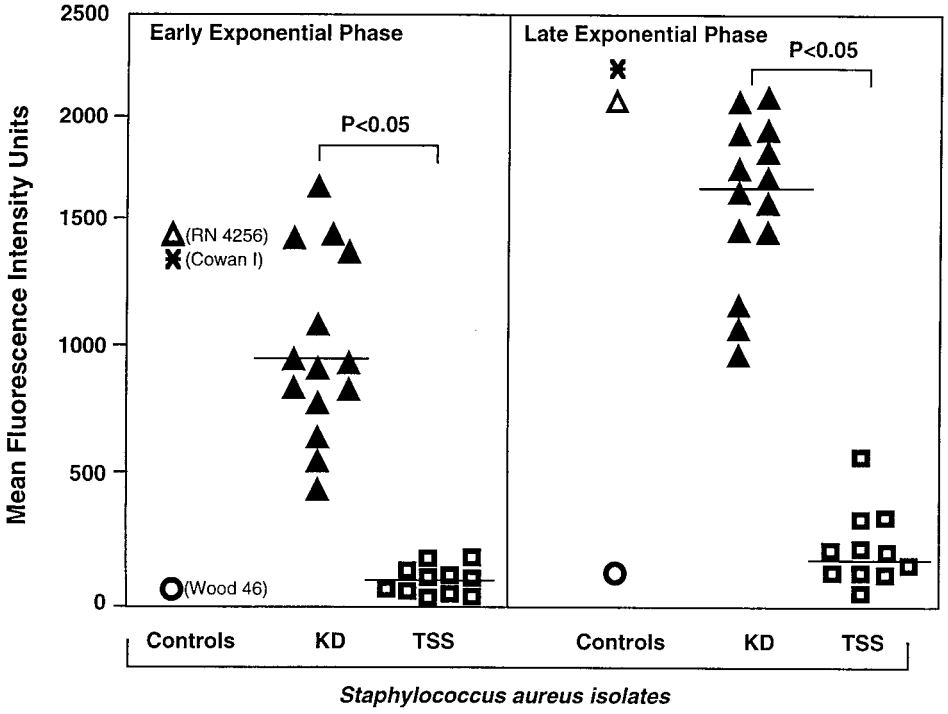

FIG. 2. Cell wall-associated SpA expression by KD and TSS isolates of *S. aureus*. Relative expression of SpA was determined using flow cytometry by measuring the MFI after incubation with biotinylated anti-SpA MAb and phycoerythrin-conjugated streptavidin, as described in the text. Each value represents the mean of duplicate analyses on the same isolate during the early and late exponential growth phases (t_1 and t_2 in Fig. 1A, respectively). Strains RN4256, Cowan I, and Wood 46 were included in the study as controls. KD isolates were found to express significantly ($P < 0.05$) more SpA on their cell surface than TSS isolates as calculated by Student's *t* test.

individual isolates, ranging from 0.25 to 30.0 ng/ml. Furthermore, in contrast to cell wall-associated or extracellular SpA, there was no statistically significant difference in the levels of TSST-1 secreted by the KD or TSS groups of isolates at this growth phase (8.55 ± 5.41 and 10.00 ± 9.39 ng/ml, respectively).

Analysis of *spa* and RNAIII transcript levels. To investigate the possibility that KD isolates may have a partially defective *agr* locus, the levels of *spa* and RNAIII transcripts in early- and late-exponential-phase cultures (Fig. 1A, t_1 and t_2, respectively) of the KD ($n = 9$) and TSS ($n = 11$) isolates were compared by Northern hybridization analysis. Strains Cowan I, Wood 46, and RN4282 and its isogenic, *agr*-negative mutant, RN4256, were included in the study as controls. The results obtained for some of the isolates analyzed are shown in Fig. 1B and 1C. High levels of *spa* mRNA (ca. 1.8 kb) were detected at both growth phases in all the KD isolates analyzed (Fig. 1B, lanes 3 to 7, and data not shown), strain RN4256 (Fig. 1B, lane 2), and strain Cowan I (Fig. 1B, lane 12). This is consistent with the finding that these strains express high levels of SpA. A similar result was observed for a few TSS isolates (Fig. 1B, lanes 10 and 11, and data not shown). These TSS isolates expressed high levels of SpA, comparable to the KD isolates. The *spa* mRNA was undetectable or barely detectable in the remaining TSS isolates analyzed (Fig. 1B, lanes 8 and 9, and data not shown), strain RN4282 (Fig. 1B, lane 1), and strain Wood 46 (Fig. 1B, lane 13). This is consistent with the observation that SpA is expressed at significantly lower levels by these strains.

The RNAIII transcript (ca. 510 bp) was detected in both early- and late-exponential-phase cultures of all the strains analyzed, except for strains RN4256 and Cowan I (Fig. 1C and data not shown). This is consistent with strain RN4256 being an *agr*-defective mutant. It also suggests that Cowan I has an uncharacterized *agr* defect, which may explain the high level of SpA expressed by this strain. The RNAIII transcript was more abundant in the late-exponential-phase cultures of all the isolates tested, consistent with previous reports on the regulation of the expression of this factor. However, significantly less RNAIII was detected in the early-exponential-phase cultures of all the KD isolates and a few of the TSS isolates than of strains RN4282, Wood 46, and the remaining TSS isolates. Furthermore, an inverse correlation was observed between the levels of the RNAIII and *spa* transcripts detected for each strain at this growth phase. This is consistent with RNAIII functioning as a negative regulator of *spa* gene transcription. Interestingly, despite the elevated levels of RNAIII in the late-exponential-phase cultures of the KD isolates and certain TSS isolates, the amount of *spa* mRNA did not decrease dramatically in these isolates.

DNA sequence analysis of the RNAIII-coding region. Although all the KD isolates analyzed express RNAIII, it is possible that this transcript may not be functional in these strains. To address this question, the DNA sequence of a ca. 860-bp chromosomal fragment encompassing the RNAIII reading frame, the RNAIII P3 promoter, and the *agr* operon P2 promoter of five KD isolates and strains MN8 and FRI 1169 (TSS isolates) was determined. Each sequence was identical except that for strain FRI 1169 (data not shown). The sequences were compared to that published for strain 8325-4 (GenBank accession no. X17301), a well-characterized Agr^+ strain. The FRI 1169 sequence was identical to this except for a single nucleotide substitution 2 nt upstream from the RNAIII transcriptional start site. The sequences of the other

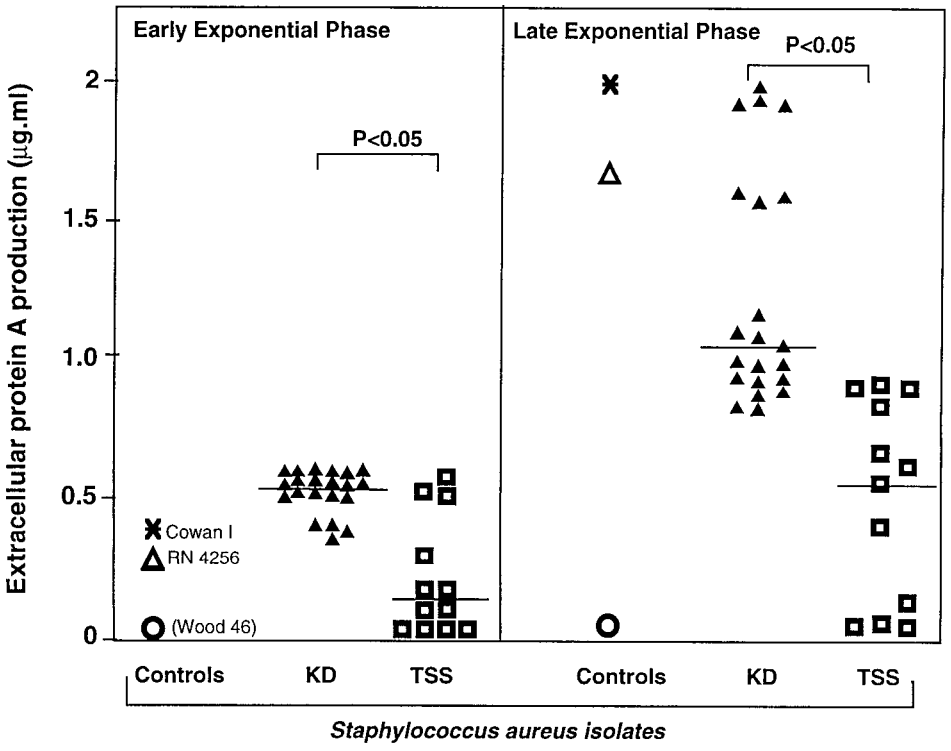

FIG. 3. Extracellular SpA secretion by KD and TSS isolates of *S. aureus*. The concentration of extracellular SpA in the supernatants of cultures grown to early and late exponential phases (t_1 and t_2 in Fig. 1A, respectively) was determined by ELISA with biotinylated anti-SpA MAb, as described in the text. Each value represents the mean of duplicate analyses of the same isolate. Strains RN4256, Cowan I, and Wood 46 were included in the study as controls. KD isolates were found to produce significantly ($P < 0.05$) more extracellular SpA than TSS isolates as calculated by Student's *t* test.

strains differed from that of strain 8325-4 in 10 nucleotide positions. However, all of these substitutions were outside the RNAIII-coding region and the −10 and −35 boxes of both the P2 and P3 promoters. There were also two insertions in the MN8/KD isolate sequence compared to strain 8325-4. A single nucleotide insertion which was not predicted to affect the RNAIII secondary structure was found at the 3′ end of the RNAIII-coding region (but outside the *hld* gene), and a 4-nt insertion was found just downstream from the RNAIII transcriptional termination site. Therefore, the sequence of the RNAIII molecule appears to be highly conserved in all the strains analyzed, with no apparent mutations that might affect the functionality or transcription level of the molecule.

DISCUSSION

It has been previously reported that TSST-1-secreting strains of *S. aureus* isolated from patients with KD exhibit an unusual phenotype which distinguishes them from TSS isolates, including the expression of lower levels of hemolyins, lipase, and protease (34). In this study, we describe another phenotype associated with KD isolates. When considered collectively, these isolates were found to express significantly more cell wall-associated and extracellular SpA than isolates from patients with TSS. This correlated with a higher level of *spa* mRNA expression in the KD isolates. Nonetheless, when considered individually, a few TSS isolates which expressed levels of *spa* mRNA and SpA comparable to the KD isolates were observed. Thus, high levels of SpA expression are not unique to the KD isolates but do appear to be a characteristic phenotype of these strains. In contrast, the KD and TSS isolates expressed comparable levels of TSST-1 in this study, consistent with previous findings (34).

It was recently suggested that the KD isolates might have a defective *agr* regulatory system that may be responsible for the usual phenotype associated with these strains (34). The high levels of SpA expressed by the KD isolates in this study are also typical of *agr*-defective mutants. Interestingly, the *agr* locus is genetically unstable in vitro (4, 37). Therefore, in this study, we chose to investigate the possibility that the KD isolates may have an *agr* defect. The *agr* locus consists of two divergent transcripts, RNAII, which contains *agrB*, *-D*, *-C*, and *-A*, and RNAIII, the effector molecule of the regulatory system (20, 41). The transcription of RNAIII is regulated by the *agr* system itself, and RNAIII is not transcribed in mutants that have a defect in any of the *agr* genes (40). In this study, all of the isolates analyzed expressed RNAIII, suggesting that all of the *agr* genes are functional. In addition, nucleotide sequence analysis of the RNAIII-coding regions of five representative KD isolates suggested that the RNAIII molecules were not mutated in these strains. Taken together, these results suggest that the KD isolates are not bona fide *agr*-defective mutants.

In this study, a correlation was observed between the levels of RNAIII and *spa* mRNA detected in each isolate in the early exponential phase (Fig. 1B and 1C, t_1). In the TSS isolates which produced low levels of *spa* mRNA and SpA protein, high levels of RNAIII were detected (Fig. 1B and 1C, lanes 8 and 9, and data not shown). This suggests that the high levels of RNAIII in these isolates may be suppressing the transcription of the *spa* mRNA. In contrast, high levels of *spa* mRNA were detected in early-exponential-phase cultures of the KD isolates and in the few TSS isolates that expressed high levels of SpA

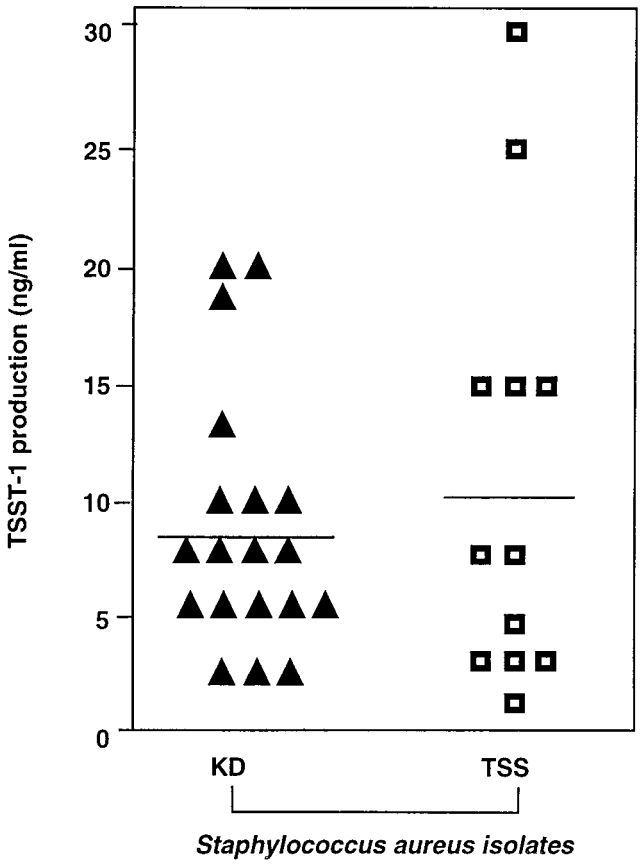

FIG. 4. Extracellular TSST-1 secretion by KD and TSS isolates of *S. aureus*. The concentration of TSST-1 in the supernatants of cultures grown to late exponential phase (t_2 in Fig. 1A) was determined by ELISA with anti-TSST-1 MAb, as described in the text. Each value represents the mean of duplicate analyses of the same isolate. KD and TSS isolates secreted similar amounts of TSST-1.

(Fig. 1B and 1C, lanes 3 to 7, 10, and 11, and data not shown). Furthermore, despite an obvious increase in the amounts of RNAIII in the late-exponential-phase cultures of these isolates, the amount of *spa* mRNA detected did not decrease dramatically. This observation differs from the results of previous studies in which the *spa* mRNA was undetectable immediately following the induction of RNAIII (47). The reason for this discrepancy is unknown. How RNAIII suppresses *spa* gene transcription has not been determined. It is possible that RNAIII indirectly regulates this and other genes by its effect on an as-yet-unidentified regulatory gene(s). As the RNAIII expressed by the KD isolates appears to be intact (based on the DNA sequence of the RNAIII-coding region), it is possible that there may be a defect in one of these downstream regulatory genes such that *spa* mRNA transcription is not suppressed in these strains. Alternatively, there may be a mutation(s) in the *spa* promoter region that makes it less susceptible to the activity of these negative regulators.

It should also be pointed out that other *trans*-acting regulatory loci may be involved in determining the level at which the *spa* mRNA is transcribed or translated (7, 9, 16). In fact, it has recently been reported that the *sar* regulatory locus suppresses *spa* mRNA transcription by *agr*-dependent and *agr*-independent mechanisms (6). The SarA protein is an activator of RNAII and RNAIII transcription, binding to a nucleotide sequence between the P2 and P3 promoters of the *agr* locus (8, 10, 19). Thus, by optimizing the transcription of RNAIII, SarA indirectly suppresses *spa* mRNA transcription. How the *sar* locus suppresses *spa* mRNA transcription independently of *agr* has not yet been determined. Given that in *sarA*-defective mutants protease expression is increased and RNAIII transcription is greatly reduced or absent (5, 8, 10, 19), it seems unlikely that the KD isolates are defective in the *sar* regulatory locus.

It is clear from this and previous studies that the KD isolates have similar phenotypes (34). In addition, our preliminary observations suggest that these isolates may also be very similar at the genotypic level. We have noted that the chromosomal restriction enzyme digestion profiles of these isolates are very similar, if not identical. Furthermore, Southern hybridization and PCR analyses of the *spa* and coagulase loci of these strains suggest that they are highly conserved, encoding five N-terminal IgG-binding repeats and four C-terminal fibrinogen-binding repeats, respectively (unpublished data). In addition, the nucleotide sequence of a ca. 860-bp chromosomal region encompassing the RNAIII-coding region and flanking DNA was identical in the KD isolates analyzed. Indeed, it is tempting to speculate that the KD isolates may be clonal in origin, as is the case for TSS isolates (39). The KD isolates used in this study were collected from a variety of geographical locations within the United States. A multicenter epidemiological study of KD isolates using traditional typing methods, such as restriction fragment length polymorphism and multilocus enzyme electrophoresis analysis, or newer typing methods based on DNA sequence analysis is warranted to further investigate the clonality of these isolates.

It is a tantalizing possibility that the expression of high levels of extracellular SpA, secreted locally by *S. aureus* isolates colonizing the gastrointestinal tract of patients, may contribute to the symptoms of KD. It has been recently demonstrated that, in addition to exhibiting immunoregulatory activities, SpA has the ability to bind to von Willebrand factor, a protein that has a central role in hemostasis and thrombogenesis (18). This raises the exciting possibility that, in addition to inducing immune activation, SpA could perturb host hemostasis and increase the risk of vascular thrombosis, such as that associated with KD. Interestingly, it has been noted that coronary complications develop in some patients diagnosed with TSS (12, 49). Whether these patients are infected with *S. aureus* strains that secrete high levels of SpA remains to be determined. Significantly, such TSS isolates were observed in this study. However, additional bacterial and host factors are likely to be involved in determining whether a patient develops classical TSS or KD (35). Investigation of the potential role of SpA in the development of KD will require an animal model of this disease in which the contribution of this and other factors can be analyzed.

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