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Relationship of *agr* Expression and Function with Virulence and Vancomycin Treatment Outcomes in Experimental Endocarditis Due to Methicillin-Resistant *Staphylococcus aureus*▽

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The accessory gene regulator (*agr*) locus has been shown to be important for virulence in several animal models of *Staphylococcus aureus* infection. However, the role of *agr* in human infections, and specifically in antibiotic treatment, is controversial. Interestingly, *agr* dysfunction has been associated with reduced vancomycin responses. To systematically investigate the role of *agr* in virulence and treatment outcome in the context of endovascular infection, 10 well-characterized vancomycin-susceptible methicillin-resistant *S. aureus* (MRSA) bloodstream isolates (5 *agr-I* [clonal complex 45, or CC45] and 5 *agr-II* [CC5]) were studied for (i) *agr* function, (ii) RNAIII transcriptional profiles, (iii) *agr* locus sequences, (iv) intrinsic virulence and responses to vancomycin therapy in an experimental infective endocarditis (IE) model, and (v) *in vivo* RNAIII expression. Significant differences in *agr* function (determined by delta-hemolysin activity) correlated with the time point of RNAIII transcription (earlier RNAIII onset equals increased *agr* function). Unexpectedly, four MRSA strains with strong delta-hemolysin activities exhibited significant resistance to vancomycin treatment in experimental IE. In contrast, five of six MRSA strains with weak or no delta-hemolysin activity were highly susceptible to vancomycin therapy in the IE model. *agr* sequence analyses showed no common single-nucleotide polymorphism predictive of *agr* functionality. *In vivo* RNAIII expression in cardiac vegetations did not correlate with virulence or vancomycin treatment outcomes in the IE model. Inactivation of *agr* in two strains with strong delta-hemolysin activity did not affect virulence or the *in vivo* efficacy of vancomycin. Our findings suggest that *agr* dysfunction does not correlate with vancomycin treatment failures in this experimental IE model in two distinct MRSA genetic backgrounds.

Staphylococcus aureus continues to be a predominant cause of community-acquired and health care-associated infections (14). It is the most prevalent cause of endovascular infections, including catheter sepsis and infective endocarditis (IE) (8, 46), and the second most common cause of bacteremia (8, 16). The increase in infections due to methicillin-resistant *S. aureus* (MRSA), the high rates of vancomycin clinical treatment failures, and the growing problems of linezolid and daptomycin resistance have all further complicated the management of patients with MRSA infections and have led to high health care costs (2, 9, 15, 30). In addition, MRSA bacteremia and IE are associated with high rates of morbidity and mortality (15 to 40%), even with seemingly appropriate antimicrobial therapy (9, 20).

There are a number of factors that appear to play key roles in the virulence of *S. aureus* infections. The accessory gene regulator (*agr*) locus, a well-characterized quorum-sensing two-component regulatory system, is a principal global regulator within the overall staphylococcal virulon. *In vitro*, *agr* upregulates secreted proteins and downregulates many pivotal surface adhesins during postexponential growth. The *agr* locus is complex, consisting of two divergent transcription units, driven by promoters P2 and P3 (reviewed in reference 34). The P2 operon encodes a two-component signaling module, of which AgrC is the receptor and AgrA is the response regulator. It also encodes two proteins, AgrB and -D, which combine to produce and secrete an autoinducing peptide (AIP) that is the ligand for AgrC. AgrA activates the *agr* P3 promoter, which drives the synthesis of RNAIII, the effector of target gene regulation. RNAIII also encodes delta-hemolysin, which makes semiquantitative scoring of delta-hemolysin elaboration a commonly used indicator of RNAIII expression and, thus, *agr* function (42, 43). There are four types of *agr* quorum-sensing systems (18, 19). Each of these *agr* systems, referred to as *agr-I* through *agr-IV*, recognizes a unique AIP structure (AIP-I through AIP-IV). Several distinct animal models have demonstrated that the *agr* system plays an important role in the infectious process, including arthritis (1), subcutaneous abscesses, mastitis, IE (3), and osteomyelitis (12); *agr* mutants have been shown to be attenuated in virulence in all these models.

There have been numerous reports suggesting that treatment of MRSA infections with vancomycin can be ineffective, despite *in vitro* susceptibility of the organism to this agent (11, 28, 29, 31). There are several potential mechanisms that have

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TABLE 1. Strains used in this study

Source and strain	Relevant characteristics[a]	VAN MIC (μg/ml)[b]	Reference(s)
Laboratory strains			
RN4220	NCTC8325-4, α-hemolysin negative, β-hemolysin positive		33
RN6911	NCTC8325-4 *agr*::*tet*(M), Tc^r		33
SH1000	*rsbU*-positive derivative of NCTC8325-4, *agr-I*		17
SH1001	SH1000 *agr*::*tet*(M), Tc^r		17
Clinical MRSA bacteremia isolates			
300-087	*agr-I*, SCC*mec* IV, CC45[c]	0.5	7, 37
324-136	*agr-I*, SCC*mec* IV, CC45[c]	0.5	7, 37
300-169	*agr-I*, SCC*mec* IV, CC45[c]	0.5	7, 37
300-103	*agr-I*, SCC*mec* IV, CC45[c]	0.5	7, 37
301-188	*agr-I*, SCC*mec* IV, CC45[c]	0.5	7, 37
300-246	*agr-II*, SCC*mec* I, CC5[c]	0.5	7, 37
010-016	*agr-II*, SCC*mec* II, CC5[c]	1	7, 37
077-107	*agr-II*, SCC*mec* II, CC5[c]	1	7, 37
088-180	*agr-II*, SCC*mec* II, CC5[c]	0.5	7, 37
088-237	*agr-II*, SCC*mec* II, CC5[c]	0.5	7, 37
Mutants			
300-169Δ*agr*	300-169 *agr*::*tet*(M), Tc^r	0.5	This study
324-136Δ*agr*	324-136 *agr*::*tet*(M), Tc^r	0.5	This study

[a] Tc^r, tetracycline resistant.
[b] Vancomycin MICs were determined according to CLSI guidelines.
[c] *spa* types were used to predict clonal complexes, according to the methods of McCalla et al. (24).

been coassociated with reduced vancomycin treatment success in both human and experimental staphylococcal infections, such as reduced susceptibility to host defense cationic peptides (9, 40) and increased biofilm formation (40). Interestingly, attenuated *agr* function, especially in association with *agr-II* strains, and development of glycopeptide intermediate resistance *S. aureus* (GISA) in association with the *agr-II* genotype have both been associated with reduced vancomycin responses (9, 27, 37, 38). To more systematically evaluate the role of the *agr* locus in virulence and vancomycin treatment outcome, we studied the composite correlations of *agr* activation, functionality, locus sequence, intrinsic virulence, and vancomycin treatment outcomes in a relevant animal model of endovascular infection by using 10 recent clinical MRSA bloodstream isolates and two *agr* mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Ten MRSA bloodstream isolates were selected from a recent strain collection of 36 MRSA strains (Table 1) (7, 40). Because *agr-I* comprises a majority of current *S. aureus* clinical scenarios (44) and *agr-II* MRSA strains are putatively associated with vancomycin therapy failure in the clinical setting (31), we focused on isolates with these two *agr* types (Table 1). Within the *agr-I* group, we selected five isolates genotyped as clonal complex 45 (CC45), because this genetic background has been associated with reduced vancomycin responses clinically (11). Among the *agr-II* isolates in the overall strain collection, all were genotyped as CC5, the most common CC type in MRSA bacteremia and which has been associated with complicated MRSA infections (10, 24). Five isolates were randomly selected from the latter CC group. The overall clinical and demographic characteristics of the patient sources of these 36 strains in this collection have been previously detailed (7).

Phage ϕ11 was used to transduce the *agr*::*tet*(M) mutation from *S. aureus* strain RN6911 into two *agr-I* (CC45) strains (300-169 and 324-136) (33). The generated strains, 300-169Δ*agr* and 324-136Δ*agr*, had a complete deletion of the *agr* locus. The *agr* locus deletions were confirmed by Southern blot analysis according to standard protocols using RNAIII-specific digoxigenin (DIG)-labeled probes produced by using the PCR DIG probe synthesis kit (Roche, Basel, Switzerland) and RNAIII-specific primers (Table 2). *S. aureus* was routinely grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) plates with 5 μg/ml tetracycline for mutant selection.

Determination of MICs, kill curve experiments, and population analyses. Determination of vancomycin MICs were conducted by broth microdilution as recommended by the CLSI (4). *In vitro* vancomycin kill curves and vancomycin population analyses were carried out as previously described (6, 48). All experiments were performed at least twice for each strain on different days.

Assessment of delta-hemolysin activity. Delta-hemolytic activities were determined by cross-streaking test strains perpendicularly to RN4220, a strain which is a hyperproducer of beta-hemolysin but does not produce alpha-hemolysin (42, 43). Strains SH1000 and SH1001 (*agr* mutant of SH1000) were used as positive and negative controls, respectively (Table 1). Delta-hemolytic activity was denoted by an enhanced area of hemolysis at the intersection of RN4220 and test strain streaks. All experiments were conducted at least three times on separate days.

Northern blot analyses. Overnight cultures of *S. aureus* were diluted to ~10^7 CFU/ml (optical density at 600 nm [OD_{600}], 0.05; dilution factor, >1:200) in fresh TSB medium and grown at 37°C in a shaking incubator. Samples were removed at 2, 4, 6, and 8 h of growth. RNA extraction and Northern blotting were performed as previously described (41). Digoxigenin-labeled DNA probes were used for the detection of RNAIII, *spa*, and *hla* transcripts by Northern hybridization. Primers used for probe amplification are listed in Table 2. Northern blot analyses were performed with samples from three different days.

Quantitative RT-PCR. To confirm RNAIII expression levels at the early time point, RNAIII transcripts from 2-h samples (OD_{600}, 0.6 ± 0.05 [mean ± standard deviation, SD] for all tested strains) were quantified by quantitative reverse transcription-PCR (RT-PCR). In addition, RNA was isolated from *in vitro* cultures that were grown for 24 h in order to compare *in vivo* cardiac vegetation samples collected 24 h postinfection (see below). RNA samples were treated with Turbo DNA free (Ambion) for 30 min at 37°C. DNase treatment was stopped using DNase inactivation reagent (Ambion). Two micrograms of RNA was transcribed into cDNA by using a RETROscript kit and 5 μM random decamer primers (Ambion). Quantitative real-time PCR was carried out using an ABI

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)	Purpose	Reference
RNAIII+	AGATCACAGAGATGTGATGG	Northern/Southern blotting	41
RNAIII−	TCGATGTTGTTTACGATAGC	Northern/Southern blotting	41
spa-F	TGAATTCGTAAACTAGGTGTAGG	Northern blotting	41
spa-R	CGGTACCAGGCTTGTTATTGTCTTCC	Northern blotting	41
hla+	AAGGTACAGTTGCAACTACC	Northern blotting	41
hla−	ATAACTGTAGCGAAGTCTGG	Northern blotting	41
gyrB.MB-F2	CGCAGGCGATTTTACCATTA	RT-PCR	This study
gyrB.MB-R2	GCTTTCGCTAGATCAAAGTCG	RT-PCR	This study
RNA3.MB-F	GCCATCCCAACTTAATAACCA	RT-PCR	This study
RNA3.MB-R	TGTTGTTTACGATAGCTTACATGC	RT-PCR	This study

Prism 7000 instrument (Applied Biosystems) and the SYBR green PCR master mix (Applied Biosystems). Reaction mixtures were prepared using 100 nM primers listed in Table 2. *gyrB* was used to normalize for transcript quantification, because it has been shown to be not affected by cell density and/or *agr* (13). Relative RNAIII expression was calculated as the differences in cycle thresholds (ΔC_T) (*gyrB* C_T − RNAIII C_T) for all samples as previously described (25, 47, 53). PCR experiments were performed using two biological replicates, each tested in triplicate.

DNA isolation and *agr* locus sequencing. Single colonies of *S. aureus* were isolated and restreaked onto TSA plates and grown overnight at 37°C. Chromosomal DNA was isolated using a Wizard genomic DNA purification kit (Promega, Madison, WI), with 20 μg/ml lysostaphin (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. The *agr* loci were PCR amplified using a method published previously (42). Both strands of the PCR product were commercially sequenced by the standard Sanger dideoxynucleotide method. DNA sequences were assembled and analyzed using the DNAStar (Madison, WI) sequence analysis suite. Each *agr* sequence was compared to a prototypical strain with each specific *agr* genotype (23, 32).

Experimental rabbit IE model. Rabbits were maintained in accordance with the American Association for Accreditation of Laboratory Animal Care criteria. The Animal Research Committee (IACUC) of the Los Angeles Biomedical Research Institute at Harbor—UCLA Medical Center approved all animal studies. A model of left-sided catheter-induced IE in rabbits was used to study composite virulence and responsiveness to vancomycin therapy among the study MRSA strains (51). Briefly, female New Zealand White rabbits (Harlan Laboratories; 2.0 to 2.5 kg body weight) underwent transcarotid-transaortic valve catheterization, and IE was induced by intravenous (i.v.) injection of ~10^5 CFU of each MRSA strain at 24 h after catheterization. At 24 h after infection, animals were euthanized by a rapid intravenous injection of sodium pentobarbital (100 mg/kg; Abbott Laboratories), and their cardiac vegetations, kidneys, and spleen were removed and quantitatively cultured (51). Tissue MRSA counts are given as the mean $\log_{10}$ CFU/g of tissue.

To assess responses to vancomycin therapy, at 24 h postinfection, animals with IE were randomized to receive vancomycin therapy (15 mg/kg i.v., twice per day for 3 days). This is a standard effective dose of vancomycin in the experimental IE model caused by vancomycin-susceptible strains (5, 51). At 24 h after the last vancomycin dose, animals were sacrificed, and target tissues were removed and quantitatively cultured as above.

The following definitions were used for grading the vancomycin treatment response: responders were defined as strains in which treatment led to a ≥5-$\log_{10}$ reduction in the number of CFU/g of vegetations and ≥3-$\log_{10}$ reduction in CFU/g of kidney and spleen. Nonresponders were defined as strains with <1.5-$\log_{10}$ reductions in CFU/g in all three target tissues (vegetations, kidneys, and spleen). These definitions were based on extensive pilot studies related to vancomycin responsiveness of MRSA IE in this model.

***In vivo* RNAIII expression in cardiac vegetations by quantitative RT-PCR.** At 24 h postinfection, catheterized animals were sacrificed, and cardiac vegetations were quick-frozen in liquid nitrogen. Total RNA was isolated using Tri reagent (Ambion) according to the manufacturer's instructions, including a ¼-in. ceramic sphere in the cell disruption step. Samples were treated with DNase, and 2 μg of total *in vivo* RNA was transcribed into cDNA as described above. Quantitative RT-PCR analysis for RNAIII expression was performed on at least two different animals for each strain.

Statistical analysis. Means and SDs were calculated for MRSA tissue counts and relative RNAIII expression levels. To compare tissue MRSA counts of treated versus untreated animals and for the analysis of the relationship between RNAIII expression levels and vancomycin responses (responders versus nonresponders), univariate analyses were performed using Student's *t* test. To assess the relationship between RNAIII expression levels and virulence in terms of bacterial density in target tissues, univariate analyses were performed with simple linear regression. *P* values of <0.05 were considered statistically significant.

RESULTS

***In vitro* susceptibility to vancomcyin.** All 10 MRSA isolates were susceptible to vancomycin, with MICs of 0.5 or 1.0 μg/ml (Table 1). No strain exhibited vancomycin tolerance based on *in vitro* kill curve kinetics, and population analyses revealed no evidence of vancomycin-heteroresistant subpopulations (data not shown).

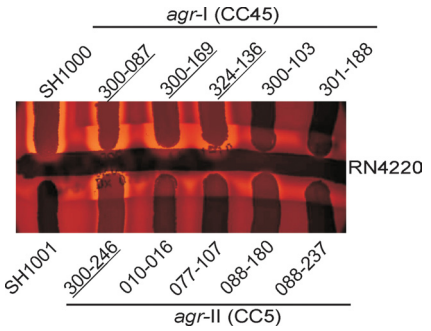

FIG. 1. Delta-hemolysin activities of 10 clinical MRSA isolates. Strains were tested against RN4220, and strains SH1000 and SH1001 (SH1000Δ*agr*) served as positive and negative controls, respectively. MRSA isolates with strong delta-hemolysin activities are underlined.

Delta-hemolysin activities and RNAIII transcription profiles. As a first screen for *agr* function, we tested the 10 clinical MRSA isolates for delta-hemolysin production. Three *agr-I* (CC45) strains and one *agr-II* (CC5) strain produced strong delta-hemolysin zones (Fig. 1). In contrast, two *agr-I* (CC45) strains and three *agr-II* (CC5) strains exhibited weak delta-hemolysin production. Strain 088-237 produced no delta-hemolysin (Fig. 1).

To further evaluate *agr* function, we assessed *agr* RNAIII expression during growth by Northern blot analyses. The four strains with strong delta-hemolysin activity exhibited a relatively early onset of RNAIII expression, beginning at 2 h of growth (Fig. 2). The other six strains had no RNAIII transcripts detectable before 4 h. Strain 088-237 exhibited no RNAIII transcripts at any of the time points tested, compatible with the delta-hemolysin-negative phenotype of this strain. These data indicated that early onset of RNAIII transcription correlated with strong delta-hemolysin production, independent of the *agr* genotype. Growth rates for all 10 strains were similar, suggesting that the observed differences in RNAIII expression onset were not due to differences in growth dynamics (data not shown). To exclude any differences in processing (e.g., blotting time, film exposure time) that might lead to differences in transcript detection, quantitative RT-PCR of the 2-h samples was carried out (Fig. 3A). Within the *agr-I* (CC45) group, the strains with strong delta-hemolysin production (300-087, 300-169, and 324-136) exhibited significantly higher RNAIII transcript levels than the other strains at the 2-h time point. Accordingly, within the *agr-II* (CC5) group, strain 300-246, which exhibited strong delta-hemolysin activity, showed the strongest RNAIII transcription at this time point. Thus, quantitative RT-PCR results were in accordance with the Northern blotting results, as shown above (Fig. 2).

***spa* and *hla* expression profiles.** To further characterize *agr* function of the strains, we examined the expression of *spa* (encoding protein A), generally repressed by *agr*, and of *hla* (encoding alpha-toxin), generally activated by *agr* (35). In all strains with strong delta-hemolysin activity, *spa* transcripts were detectable at only 2 h but not at later time points (data not shown). Most strains with weak or no delta-hemolysin production exhibited *spa* transcripts throughout the 2- to 8-h time points. Even though strain 300-103 exhibited weak delta-hemolysin activity, it exhibited *spa* transcripts at 2 h, with only

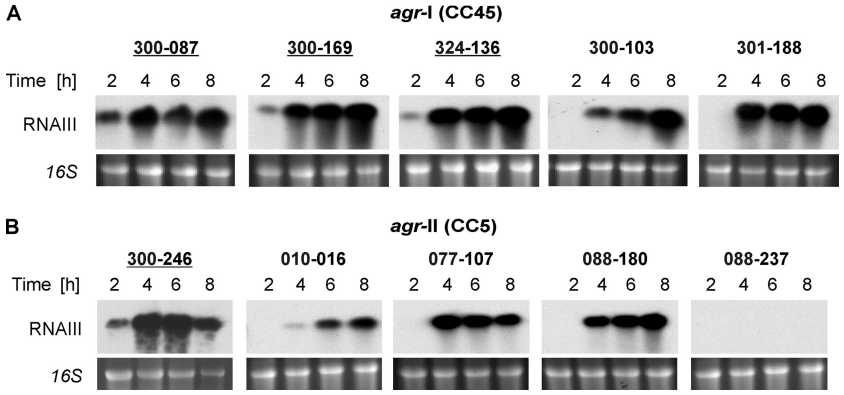

FIG. 2. Northern blot analyses of RNAIII, transcripts of five clinical MRSA isolates with an *agr-I* (CC45) background (A) and five clinical MRSA isolates with an *agr-II* (CC5) background (B). Transcripts and time points are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading. Isolates with strong delta-hemolysin activities are underlined.

smears detectable at later time points. All strains, except strains 010-016 and 088-237, expressed *hla* at 4 h and later time points. Strain 010-016 exhibited *hla* transcripts at 6 and 8 h only. In strain 088-237, only weak *hla* transcripts were detectable at 8 h, which is in accordance with the absence of RNAIII transcripts in this strain (data not shown).

***agr* locus sequence analyses.** We sequenced the entire *agr* locus of the 10 strains (including RNAII, RNAIII, and promoter regions). The *agr* sequences of all five *agr-I* (CC45) strains studied were similar to the previously published *agr* sequence of strain Germ825/96, a strain with a similar genetic background (36). Strains 300-087, 300-169, 324-136, and 301-188 had identical *agr* locus sequences, suggesting that the weak delta-hemolysin activity of 301-188 was not mediated by *agr* mutational events. The *agr* sequence of 300-103, the other *agr-I* (CC45) strain with weak delta-hemolysin activity, differed by 1 amino acid within *agrA*: V232L.

The *agr* sequences of *agr-II* (CC5) strains 010-016, 077-107, and 088-180 (all having weak delta-hemolysin activities) were identical to the *agr* sequence of strain JH1 (32). The *agr* sequence of 088-237 differed by 1 nucleotide from these three strains in *agrA* (L96S), which is a potential explanation for the lack of delta-hemolysin activity in this strain. The *agr* sequence of strain 300-246, which exhibited strong delta-hemolysin activity, differed from the other four *agr-II* (CC5) strains by 1 nucleotide within the intergenic region between RNAII and RNAIII: A→G. This mutation was located 34 bp downstream of the P2 region, and it is unclear if this mutation was the reason for the strong delta-hemolysin activity observed in this strain.

IE model. To define whether the above *in vitro agr* RNAIII transcription, *agr* functional, and whether sequence profile differences translated to infection outcomes *in vivo*, the 10 strains were studied in experimental IE for their intrinsic virulence and vancomycin responsiveness (Fig. 4). The intrinsic virulence of the study strains in the IE model (based on achievable target tissue MRSA counts) was similar among the isolates within the *agr-I* (CC45) group (Fig. 4A). Interestingly, within the *agr-II* (CC5) group, strain 088-237, a natural *agr* mutant, had significantly higher bacterial densities in vegetations and kidneys than strains 077-107 and 088-180 (Fig. 4B) ($P < 0.05$). Animals infected with *agr-I* (CC45) strains had significantly higher MRSA densities in all three target tissues 24 h after infection than animals infected with strains from the *agr-II* (CC5) group ($P < 0.05$). Overall, there was no obvious relationship between *agr* functionality and intrinsic fitness of the 10 strains.

Strains with strong delta-hemolysin activity and early RNAIII onset (e.g., 300-087, 300-169, 324-136, and 300-246) did not respond to vancomycin treatment; residual target tissue MRSA densities in vancomycin-treated animals infected with these strains were similar to those in their respective untreated control groups (Fig. 4A and B). In contrast, vancomcyin treatment of rabbits infected with five strains that exhibited weak or no delta-hemolysin activity *in vitro* resulted in uniform and highly significant reductions of MRSA counts in all target tissues compared with their respective control groups ($P < 0.001$ for all strains and all three target tissues). The only outlier was strain 300-103, which exhibited weak delta-hemolysin activity but which was not responsive to vancomycin treatment in the IE model.

RNAIII expression in cardiac vegetations. To assess whether there was a correlation between *in vivo* and *in vitro agr* activities and treatment outcomes, we quantified RNAIII expression in cardiac vegetations at 24 h postinfection. Figure 3B and **C** show RNAIII expression levels of the 10 strains grown *in vitro* for 24 h and in cardiac vegetations in the IE model 24 h postinfection, respectively. All strains except strain 088-237 exhibited high RNAIII expression levels at 24 h *in vitro*. RNAIII expression levels in cardiac vegetations in the IE model at 24 h postinfection were significantly lower than the 24-h *in vitro* samples ($P < 0.01$ for all strains except 088-237, which is a natural *agr* mutant strain and exhibited low ΔC_T values under all conditions tested). There was no correlation between RNAIII expression levels *in vivo* and *in vitro* (2 h and 24 h; $P > 0.05$). Also, there was no correlation between *in vivo* RNAIII expression levels and virulence in terms of bacterial counts in infected target tissues and/or treatment outcomes in the IE model ($P > 0.05$ for all comparisons).

Effect of *agr* deletion on virulence and vancomycin response in the IE model. To further evaluate the role of *agr* function in virulence and vancomycin responses in the IE model, the en-

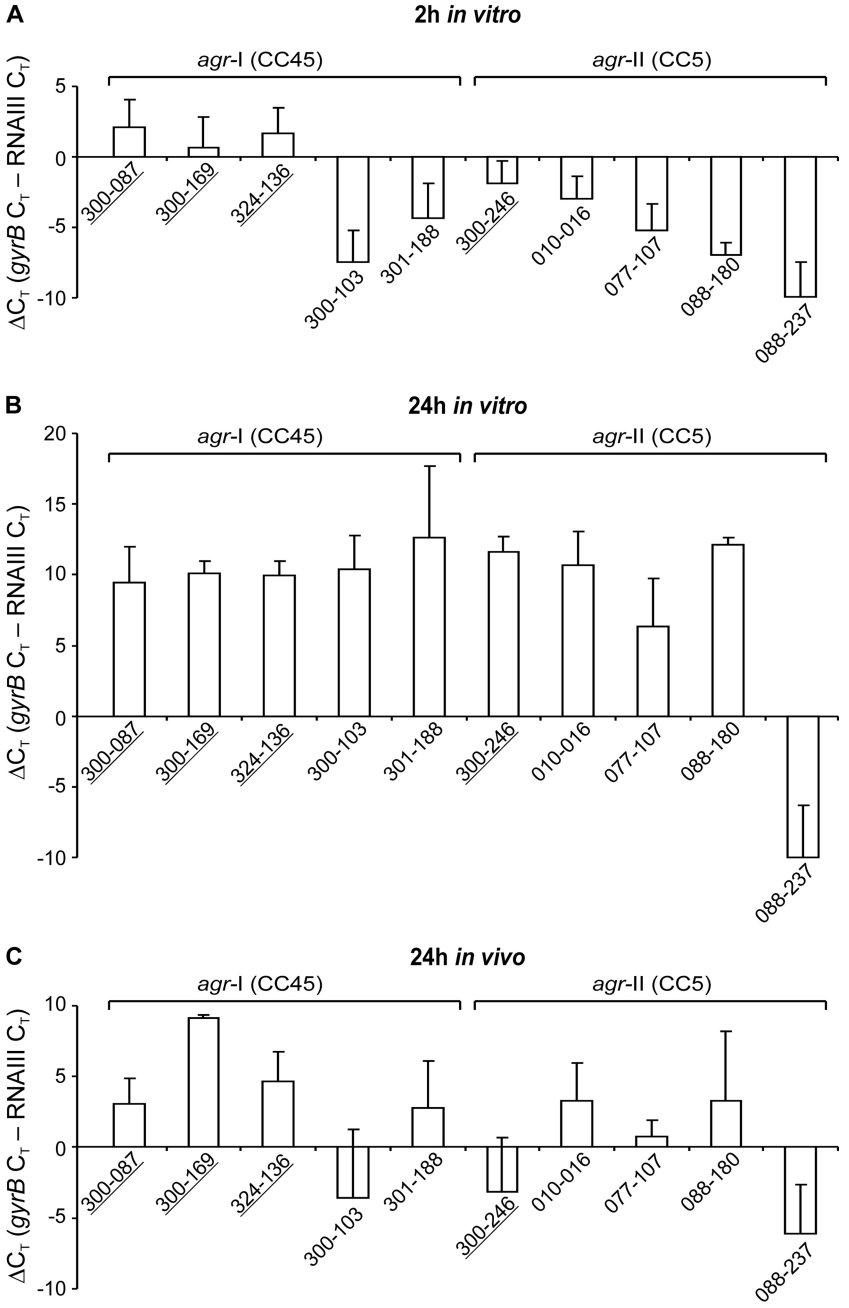

FIG. 3. Quantitative RT-PCR of RNAIII transcription at 2 h (A) and 24 h (B) *in vitro* and in infected cardiac vegetations in the IE model 24 h postinfection (C). Relative transcript levels of RNAIII were determined by RT-PCR. Results are presented as the ΔC_T (*gyrB* C_T − RNAIII C_T) and represent the mean (+SD) of two biological replicates *in vitro* and of at least two animals *in vivo*. Isolates with strong delta-hemolysin activities are underlined.

tire *agr* locus of two *agr-I* (CC45) strains that were vancomycin nonresponders in the IE model (300-169 and 324-136) was deleted. As expected, and in contrast to their parental strains, both *agr* deletion mutants exhibited strong *spa* and weak *hla* transcripts in the later growth phases (Fig. 5A). We were not successful in deleting the *agr* locus of strain 300-246, the only *agr-II* (CC5) strain that exhibited strong delta-hemolysin activity and did not respond to vancomycin treatment.

No significant changes were observed in the intrinsic virulence in these *agr* mutants (Fig. 5B) compared to their respective parental strains (Fig. 5A) in experimental IE in terms of target tissue MRSA counts ($P > 0.1$ for all comparisons). Even though statistically relevant, only relatively small reductions in target tissue counts were seen following vancomycin treatment in animals infected with 300-169Δ*agr* versus the corresponding untreated controls (<1-$\log_{10}$ CFU reduction/g of target tissue) (Fig. 5B). In animals infected with mutant strain 324-136Δ*agr*, vancomycin treatment did not significantly reduce bacterial

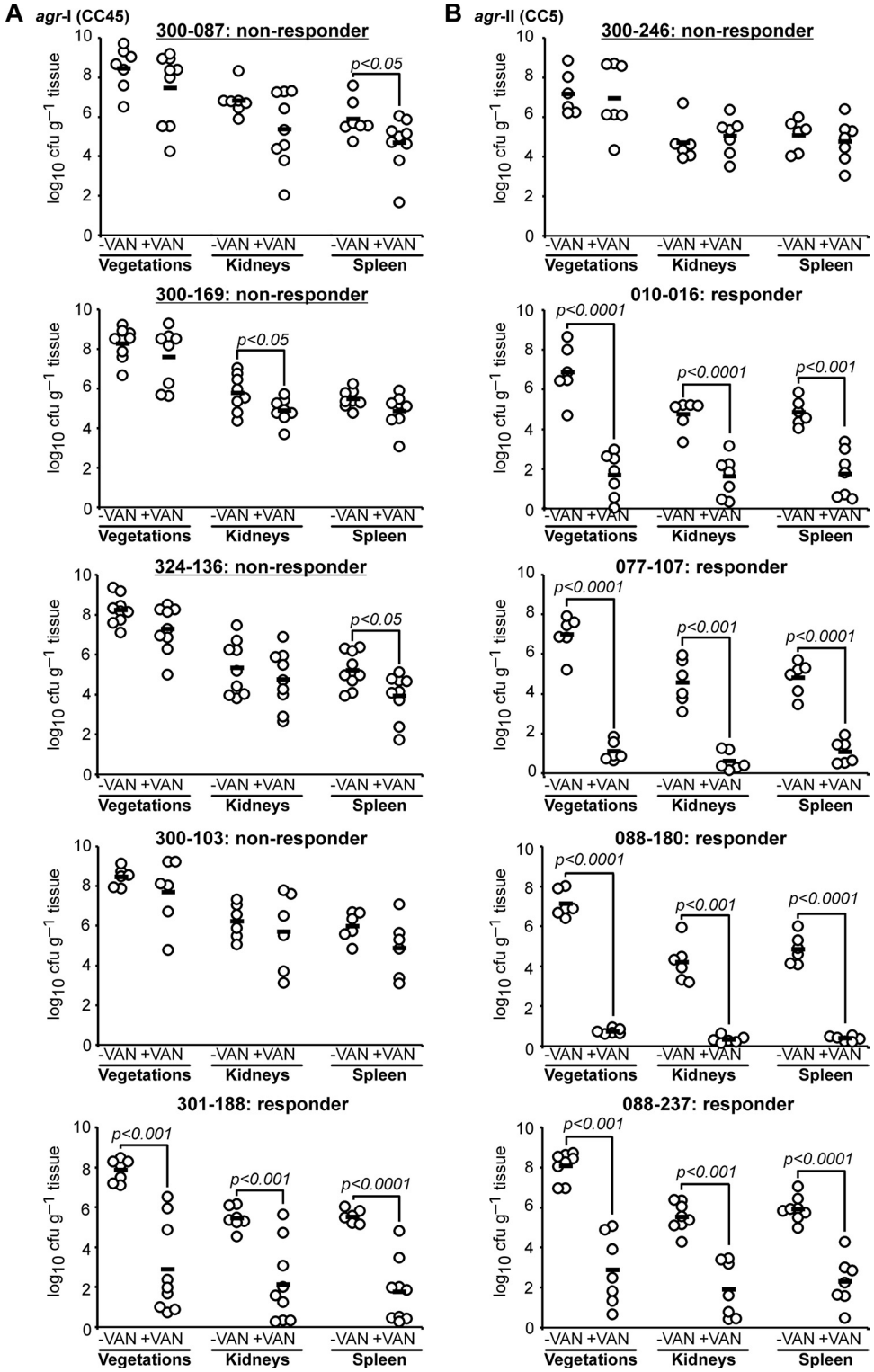

FIG. 4. Densities of MRSA in target tissues in the IE model due to 10^5-CFU challenges with and without vancomycin therapy (15 mg/kg, i.v., two times per day for 3 days, starting 24 h postinfection). (A) *agr-I* isolates; (B) *agr-II* isolates. Each dot represents one rabbit. Horizontal black bars indicate means of observations. Isolates with strong delta-hemolysin activities are underlined. A responder showed a $\geq$5-$\log_{10}$ reduction in the number of CFU per g of vegetation and $\geq$3-$\log_{10}$ reductions in CFU per g kidney and spleen due to vancomycin treatment. A nonresponder showed $<$-1.5-$\log_{10}$ reductions in CFU per g of the three target tissues (vegetations, kidneys, spleen). Isolates with strong delta-hemolysin activities are underlined.

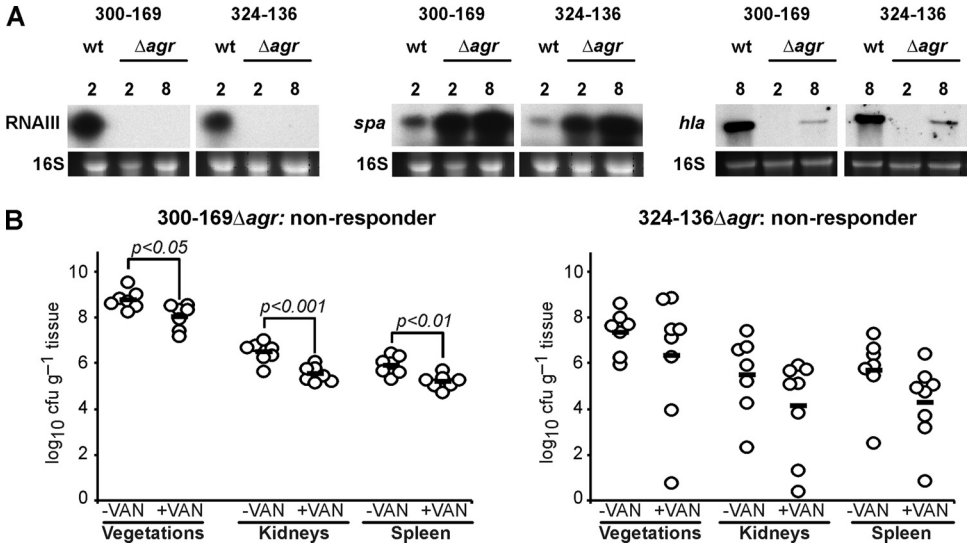

FIG. 5. (A) Northern blot analyses of RNAIII, *spa*, and *hla* transcripts of strains 300-169 and 324-136 and their *agr* mutants. Transcripts and time points are indicated, and ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading. (B) Densities of MRSA in target tissues in the IE model following inoculation of 10^5 CFU of strain 300-169Δ*agr* or 324-136Δ*agr*, with and without vancomycin therapy (15 mg/kg, i.v., twice a day for 3 days, starting 24 h postinfection). Each dot represents one rabbit. Horizontal black bars indicate means of observations. A nonresponder showed <1.5-$\log_{10}$ reductions in CFU load per g of the three target tissues (vegetations, kidneys, and spleen).

counts in any target tissue compared with untreated controls (Fig. 5B), or with its parent strain (Fig. 5A). Thus, both *agr* mutations in initially vancomycin-nonresponding strains did not alter their response profiles in the experimental IE model.

DISCUSSION

Several recent studies have reported an increased risk for vancomycin treatment failure in bacteremia associated with vancomycin-susceptible MRSA strains with phenotypic *agr* dysfunction (9, 27, 37, 38). Such *agr* dysfunction may be multifactorial, caused by mutations in the *agrA* and/or *agrC* regions of this operon that impact RNAIII transcription, function, or stability, or which inactivate delta-hemolysin translation (39, 45). In our current study, most isolates with low or no delta-hemolysin production initiated RNAIII transcription at later time points compared to isolates that exhibited strong delta-hemolysin production. Previous investigations suggested that late onset of RNAIII expression (at 4 h and later) resulted in a failure to translate delta-hemolysin and alpha-hemolysin, and hence, a weak or nonhemolytic phenotype (42, 43). Therefore, even though the lack of delta-hemolysin production is a phenotypic marker for a nonfunctional *agr* locus (9, 27, 37), its absence does not necessarily correlate with the absence of RNAIII transcripts (49).

Several studies have demonstrated that *S. aureus agr* mutants are substantially less virulent in experimental *S. aureus* infection models (1, 12, 26), and administration of an inhibitory *agr*-encoded heterotypic AIP blocked experimental murine abscess formation (50). Interestingly, one of the current study strains (088-237) was a natural *agr* mutant that did not transcribe RNAIII at any of the time points tested and did not produce delta-hemolysin. This strain was highly virulent in experimental IE, suggesting that a functional *agr* locus is not required for *in vivo* fitness in the experimental IE model. This is in accordance with previous findings (3), in which inactivation of *agr* had only minor effects on virulence in the IE model. Interestingly, the latter *agr* dysfunctional strain was highly susceptible to vancomycin treatment in the IE model. It should be emphasized that most recent studies comparing *agr* function with vancomycin treatment outcomes have been based in clinical settings and thus might well be influenced by host factors varying from patient to patient.

In the present investigation, we attempted to evaluate the role of the *agr* locus in vancomycin treatment outcomes in a relevant model of endovascular infection in which host factors were similar from animal to animal. Of note, the current study also correlated such outcomes with composite phenotypic and genotypic metrics involving *agr*, including gene transcription, functionality, sequence profiles, and *in vivo* gene expression. A number of interesting observations emerged from these investigations. Our data clearly indicate that *agr* dysfunction does not correlate with suboptimal vancomycin treatment outcomes in experimental IE in two distinct genetic backgrounds (*agr-I* [CC45] and *agr-II* [CC5]). Five of six MRSA strains tested with relatively late or no *agr* activation and weak or no delta-hemolysin production were very responsive to vancomycin therapy in experimental IE. In contrast, all four strains with strong delta-hemolysin activities did not respond to vancomycin treatment. Importantly, all isolates in this study were susceptible and nontolerant to vancomycin *in vitro* and showed no evidence of hetero-vancomycin intermediate resistance *S. aureus* subpopulations. Therefore, the different vancomycin treatment outcomes observed in the IE model were not related to any overt differences in the *in vitro* vancomycin susceptibility properties of the study strains. Interestingly, the *agr-I* (CC45) group had a higher proportion of strains that did not respond to vancomycin treatment (80%) than the *agr-II* (CC5) group (20%) in the IE model ($P < 0.05$), indicating that the vancomycin re-

sponse may be linked to specific genetic backgrounds. These findings differ from other recent clinical investigations (31), in which MRSA infections caused by *agr-II* genotype strains correlated with vancomycin treatment failure. *In vivo* and *in vitro* *agr* RNAIII expression profiles differed in the present investigation, with RNAIII transcription levels *in vivo* significantly lower than *in vitro* RNAIII expression at 24 h. This is in accordance with previous publications (13, 52), in which RNAIII expression was repressed in selected *in vivo* settings.

In order to determine whether perturbations in activation and function of *agr* might be due to acquisition of single-nucleotide polymorphisms (SNPs), we sequenced the entire *agr* loci of the 10 study strains. Our data indicated that there was no common SNP(s) linked to the temporal RNAIII expression profile within the *agr-I* (CC45) group, suggesting the possibility that loci outside *agr* contribute to the observed differences in *agr* RNAIII activation. The role of *agr* in the *in vivo* virulence of *S. aureus* is somewhat controversial. A number of recent investigations have reported that *agr* mutants reduce virulence in various animal models (1, 12, 22, 26). In contrast, other studies showed that inactivation of *agr* did not affect virulence *in vivo* various (21, 22). Therefore, to further assess the role of *agr* in vancomycin treatment responses *in vivo*, we selected two strains that did not respond to vancomycin treatment *in vivo* (300-169 and 324-136) and deleted their whole *agr* loci. Such *agr* inactivation neither reduced virulence nor significantly enhanced vancomycin responsiveness compared to the parental strains in the IE model. These data further suggest that *agr* itself does not affect virulence or vancomycin susceptibility in the IE model and that regulatory loci outside *agr* are in play in this group. The *agr* locus of the only strain with strong hemolytic activity (300-246) in the *agr-II* (CC5) group differed by 1 nucleotide from the *agr* loci of the other isolates within this group, which might explain the early *agr* activation of this strain. This strain was also the only strain in the *agr-II* (CC5) that did not respond to vancomycin treatment in the IE model. Unfortunately, we were not able to inactivate the *agr* locus of this strain. Therefore, it remains undefined as to how *agr* activity impacts treatment outcome within the *agr-II* (CC5) group. Although beyond the scope of the present study, future investigations could examine the *in vivo* impacts of *agr* knockouts in strains that respond to vancomycin treatment in the IE model.

In summary, we present here a systematic approach to assess the role of the *agr* locus in virulence and vancomycin treatment outcome in a relevant model of endovascular infection. We found that *agr* function, transcription, and sequence did not influence virulence and vancomycin treatment outcomes in the IE model among the *agr-I* (CC45) strains investigated. Studies are in progress in our laboratory to further examine the detailed roles of the *agr* locus in virulence and antibiotic treatment outcomes in the experimental IE model, using different MRSA genetic backgrounds (e.g., *agr-I* [CC22], *agr-I* [CC8], *agr-III* [CC30], and *agr-IV* [CC121]), and also more *agr-II* (CC5) isolates (including isogenic *agr* mutants).

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