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Staphylococcal Accessory Regulator (*sar*) in Conjunction with *agr* Contributes to *Staphylococcus aureus* Virulence in Endophthalmitis

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Previous studies showed that an *agr* mutant strain of *Staphylococcus aureus* was partially attenuated in virulence compared to a parental strain in experimental endophthalmitis. The purpose of this study was to determine whether the *sar* locus, either alone or through interactions with *agr*, contributes to the regulation of virulence in *S. aureus* endophthalmitis. Experimental endophthalmitis was established by the midvitreous injection of approximately 30 CFU of *S. aureus* RN6390 or the isogenic attenuated strains RN6911 (*agr* mutant), ALC136 (*sar* mutant), and ALC135 (*agr sar* double mutant). Unexpectedly, the rate of reduction in electroretinographic B-wave amplitude in eyes infected with strain ALC136 (*sar* mutant) was not significantly different from the parental strain on postinfection day (PID) 5 (10% retention). In contrast, ALC135 (*agr sar* double mutant)-infected eyes retained 73% of preoperative B-wave amplitude on PID 5. Therefore, unlike *agr*, a mutation in the *sar* locus alone does not alter the overall virulence of wild-type *S. aureus* in experimental endophthalmitis. However, the combined effect of insertional mutations in both the *sar* and *agr* global regulators leads to near-complete attenuation of virulence.

Staphylococcus aureus is a leading cause of endophthalmitis (16) and is associated with poor visual outcome (defined as final visual acuity of 20/400 or worse) in approximately 55% of reported cases (2, 18, 20, 21, 25, 28, 29, 31, 32, 35, 36, 38). The pathogenic mechanisms responsible for the poor prognosis associated with *S. aureus* endophthalmitis are not well understood. It is generally considered, however, that the major pathogenic factors in endophthalmitis are the direct toxic effects of secreted bacterial factors on intraocular tissues and indirect tissue damage resulting from the host inflammatory response. Clinical isolates of *S. aureus* secrete numerous extracellular proteins, many of which have been implicated in the pathogenesis of staphylococcal disease (10, 14, 17, 26).

Exoprotein expression by *S. aureus* is coordinately controlled by a number of chromosomally encoded global regulators which act at the transcriptional level (7, 19, 33). Accessory gene regulator (*agr*), the first staphylococcal global regulator to be identified, suppresses the post-exponential-phase expression of cell surface binding proteins (thought to play a role in the early stages of colonization) and enhances the expression of secreted proteins (suggested to be involved in the long-term survival of the organism at the site of infection) (4, 19, 30). Several experimental models of staphylococcal infection, including endocarditis, osteomyelitis, and septic arthritis, show that *agr* mutant strains are attenuated in virulence compared to corresponding parental strains, indicating that post-exponential-phase exoprotein expression is central to the pathogenesis of staphylococcal disease (1, 6, 12). In an experimental endophthalmitis model, we recently observed slower onset of retinal damage and clinical symptoms in eyes infected with an *agr* mutant strain of *S. aureus* than in eyes infected with the parental strain (5), suggesting that extracellular proteins under the control of *agr* contribute to the severity of endophthalmitis.

A second global regulatory locus, termed staphylococcal accessory regulator (*sar*), has been identified (7). Like *agr*, *sar* is also involved in the regulation of extracellular and cell wall proteins. Transposon insertional mutations within the *sar* locus result in decreased expression of both extracellular and cell surface binding proteins, such as fibronectin and fibrinogen binding proteins (6, 9). Enhanced proteolytic and lipolytic activities are also associated with a *sar* mutant genotype (6, 9, 13). Recent transcriptional and complementation analyses indicate that *sar*-encoded products interact with the *agr* locus during the mid- to late exponential phase of growth to regulate the transcription of RNAIII, the *agr* effector molecule (8, 13). Furthermore, results from animal model studies suggest that *agr* and *sar* also interact in vivo to control genes that affect the pathogenesis of *S. aureus* infection (6). Therefore, while *sar* and *agr* can differentially control exoprotein production by *S. aureus* (6, 9, 19), these global regulators also appear to interact at the DNA level to regulate exoprotein gene expression.

Based on evidence obtained from other infection models and previous findings that a Tn*551* insertional mutation in *agr* partially attenuates the virulence of *S. aureus* in experimental endophthalmitis (5), it was of interest to determine whether the *sar* locus makes an additional contribution to the expression of virulence factors by *S. aureus* during intraocular infection. We compared the relative virulence of isogenic parental, *agr* mutant, *sar* mutant, and *agr sar* double mutant strains of *S. aureus* in a rabbit model of endophthalmitis.

S. aureus RN6390 was used as the reference wild-type strain in this study (23, 27). RN6911 (24, 37), ALC136 (9), and ALC135 (6) are isogenic mutant strains of RN6390 and are

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TABLE 1. *S. aureus* strains used in this study

Strain	Genotype
RN6390	*agr*$^+$ *sar*$^+$
RN6911	*agr sar*$^+$; RN6390 with an *agr*::*tetM* insertion
ALC136	*agr*$^+$ *sar*; RN6390 with a *sar*::Tn*917LTV1* insertion (previously designated mutant R)
ALC135	*agr sar*; RN6390 with both an *agr*::*tetM* and a *sar*::Tn*917LTV1* insertion (previously designated mutant I)
ISP266	8325-4 with Tn*551* (*ery*) and *tmn-3106* (*tetM*) chromosomal insertions

described in Table 1. Detailed phenotypic characterization of each of these strains with respect to exoprotein expression has been reported previously (6, 9, 13, 19). Bacterial strains were maintained on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.). Prior to intravitreal injection, bacteria were grown for 18 h in BHI broth at 37°C. Cultures were serially diluted in sterile phosphate-buffered saline (PBS), pH 7.5. Enumeration of organisms at the time of injection and recovery from vitreous was accomplished by plating 10-μl aliquots of serial dilutions on BHI agar followed by overnight incubation at 37°C.

Growth of *S. aureus* in vitreous humor. It was previously determined that strains RN6390, RN6911, ALC136, and ALC135 had comparable growth rates in broth culture in the absence of antibiotics (6). However, to ensure that differences in virulence were not attributable to a growth advantage for the parental strain, the suitability of vitreous humor to support growth was assessed in vitro. Vitreous was recovered from normal uninfected eyes as described previously (5). Approximately 10^2 CFU of RN6390, RN6911, ALC136, or ALC135 was inoculated in triplicate into 1 ml of uninfected vitreous and incubated at 37°C for 5 days. Samples (0.02 to 0.1 ml) were taken at timed intervals after inoculation. As shown in Fig. 1, all of the strains exhibited similar growth kinetics, reaching approximately 5×10^7 CFU/ml of vitreous by 48 h ($P = 0.1109$; Kruskall-Wallis rank sums test).

Rabbit model of endophthalmitis. Forty New Zealand White rabbits weighing 2 to 4 kg were used in this study. Animals were housed and cared for at the Dean A. McGee Eye Institute animal care facility in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. One hundred microliters of *S. aureus* suspension in PBS, containing approximately 30 CFU, was slowly infused into the center of the vitreous body (RN6390, $n = 9$; ALC136, $n = 9$; RN6911, $n = 12$; ALC135, $n = 10$) as previously described (5). Contralateral eyes served as surgical controls and were injected with 100 μl of sterile PBS.

ERG. The scotopic B-wave response in infected eyes was measured by electroretinography (ERG) on postinfection days (PID) 1, 2, 3, and 5 as previously described (5). One-way analysis of variance was performed on data collected on PID 5. Fisher's least-significant-difference test was used to make pairwise comparisons between the groups, and P values of <0.05 were considered statistically significant. The results are shown in Fig. 2. On PID 1, the B-wave amplitude in parental-strain-infected eyes (76.9% of B wave retained) was only moderately reduced compared to that in eyes infected with each of the isogenic mutant strains (RN6911, 93.6%; ALC136, 89.9%; ALC135, 98.5%) as well as the uninfected surgical control eyes (87.5%). However, at PID 2 through 5, strain RN6390 caused a rapid decline in B-wave amplitude (PID 2, 19.0% retained; PID 3, 10.0%; PID 5, 6.1%). Unexpectedly, the *sar* mutant, ALC136, caused a rapid reduction in ERG B-wave amplitude similar to that caused by the parental strain (PID 2, 22.5% retained; PID 3, 13.4%; PID 5, 7.9%), and on PID 5 there was no significant difference between ALC136- and parental-strain-infected eyes ($P > 0.05$).

B-wave amplitude retention in eyes infected with strain RN6911 (*agr* mutant) was substantially higher on PID 2, 3, and 5 than in either parental-strain-infected or *sar* mutant (ALC136)-infected eyes ($P < 0.05$ on PID 5). Eyes infected

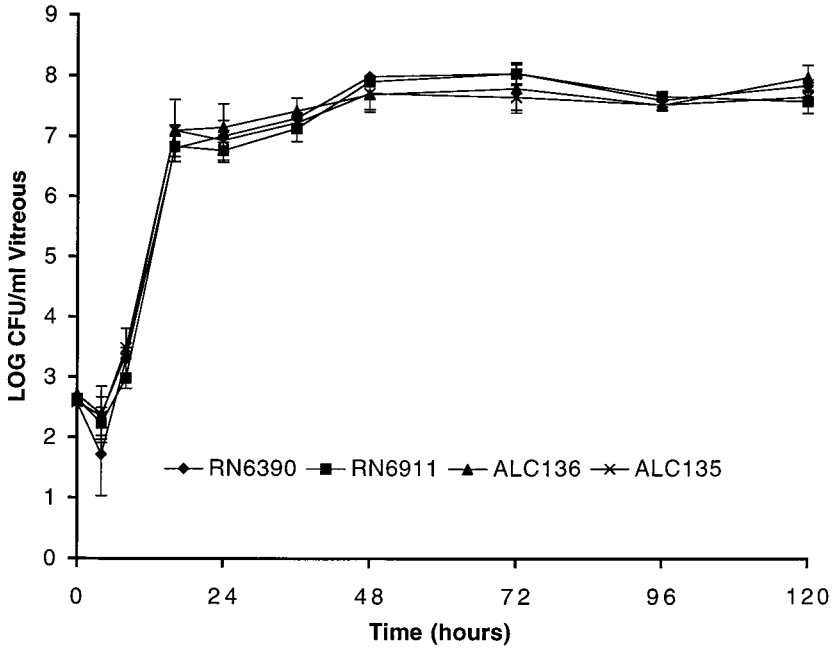

FIG. 1. In vitro growth rate of *S. aureus* RN6390 (parental strain), ALC136 (*sar* mutant), RN6911 (*agr* mutant), and ALC135 (*agr sar* double mutant) in vitreous.

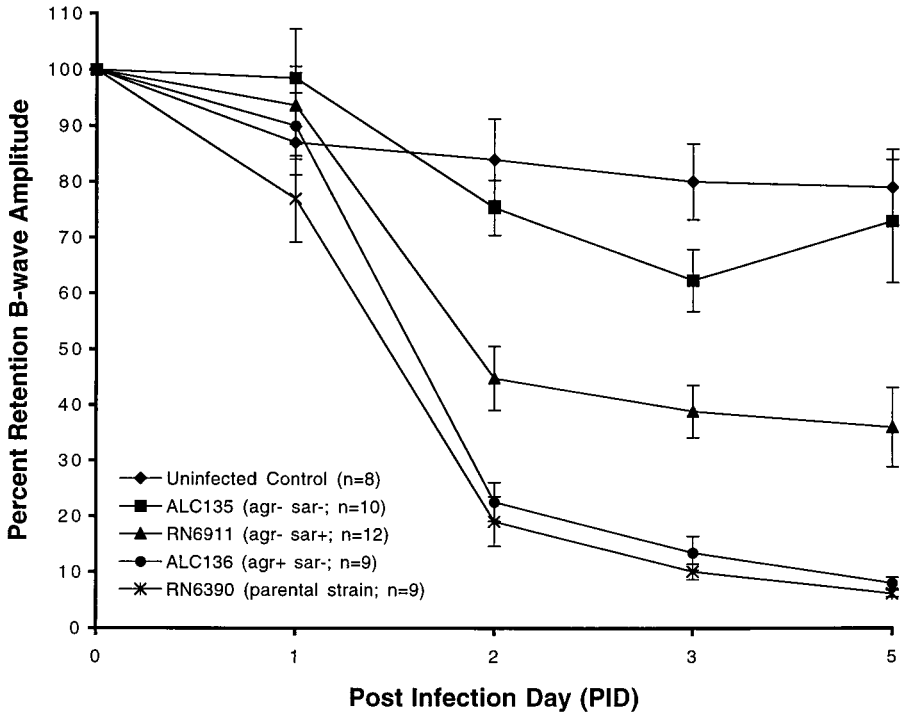

FIG. 2. B-wave amplitude in rabbit eyes infected with approximately 30 CFU of *S. aureus* RN6390 and its corresponding *agr* and/or *sar* mutant strains. Surgical control eyes were injected with 100 μl of sterile PBS. Error bars indicate the standard error of the mean B-wave amplitude. One-way analysis of variance indicated a significant difference between the groups on PID 5 ($P < 0.001$).

with the double mutant ALC135 (*agr sar*) retained a higher B-wave amplitude on PID 2, 3, and 5 than any of the other infection groups ($P < 0.05$ on PID 5). Interestingly, by PID 5, no significant difference was observed between ALC135-infected eyes and the uninfected surgical control eyes ($P > 0.05$).

To control for the presence of antibiotic resistance genes on the chromosome of the strains used in this study, *S. aureus* 8325-4 ISP266 (which carries Tn*551* [Erm^r] and *tmn-3106* [Tet^r] chromosomal insertions at sites irrelevant to any of the known global regulators or virulence determinants) was also evaluated. Strain 8325-4 ISP266 showed a temporal pattern of ERG B-wave loss similar to that of wild-type strain RN6390 (data not shown), and at PID 5 there was no significant difference between the strains ($P = 0.275$), indicating that the presence of two antibiotic resistance genes on the chromosome does not influence the virulence of the infecting organism.

Histopathological analysis. Infected eyes were enucleated for histopathological analysis on PID 1 and 3. Eyes were fixed in 10% formalin for at least 24 h, processed, sectioned, and stained with hematoxylin and eosin by standard procedures. Pathological interpretation was made with the investigator blinded as to the identity of the infecting organism. On PID 1, all infection groups showed intact retinal structure. This is consistent with normal neuroretinal responsiveness (B-wave amplitude) observed for all infection groups at this time point (Fig. 2). Eyes infected with RN6390 exhibited moderate vitritis with otherwise normal histology. Eyes injected with RN6911, *sar* mutant ALC136, and double mutant ALC135 showed no histopathologic changes (data not shown). Histopathological changes of varying severity were observed for all infection groups by PID 3 (Fig. 3). Dense fibrin deposition was observed in the anterior chamber of eyes infected with parental strain RN6390. Vitreous changes in the same eyes included marked infiltration of inflammatory cells, vitreous syneresis, and focal areas of abscess formation. Where abscess formation occurred in proximity to the retina, the inner limiting membrane was disrupted and infiltration of inflammatory cells was observed (Fig. 3A). The inner and outer nuclear layers remained intact. Moderate choroiditis with dilation of choroid vessels was also apparent. Marked fibrin deposition was observed in the anterior chamber of ALC136 (*sar* mutant)-infected eyes on PID 3, along with marked inflammatory reaction, vitreous abscess formation, and extensive vitreous liquefaction. Areas of inner limiting membrane disruption which extended to full-thickness disruption in some areas were observed (Fig. 3B). Severe choroidal inflammation was also observed. Mild anterior chamber inflammation was associated with strain RN6911 (Fig. 3C)- and double mutant ALC135 (Fig. 3D)-infected eyes. The latter infection groups exhibited moderate and mild vitritis, respectively, with intact retinal architecture and no choroiditis. However, unlike ALC135-infected eyes, RN6911-infected eyes exhibited focal areas of abscess formation located near the vitreo-retinal interface, as well as areas of retinal detachment.

SLE. Infected eyes were examined by slit lamp biomicroscopy on PID 1, 2, 3, and 5 in order to make comparative evaluations between infection groups and monitor the clinical progression of endophthalmitis. Animals underwent topical ocular anesthesia as previously described (5). Clinical observations were conducted with the investigator blinded as to the identity of the infecting organism and were graded according to the scheme outlined by Smith and Nozik (34). The parameters evaluated were anterior chamber cell, anterior chamber flare, and anterior vitreous reaction. Each parameter was graded on a scale of 0 to 4, where 4 represented the most severe reaction. The slit lamp examination (SLE) score assigned to each rabbit eye was the total score for each of the three measures (maximum possible score = 12). The Kruskall-Wallis rank sums test was used to compare the groups on PID 5, and

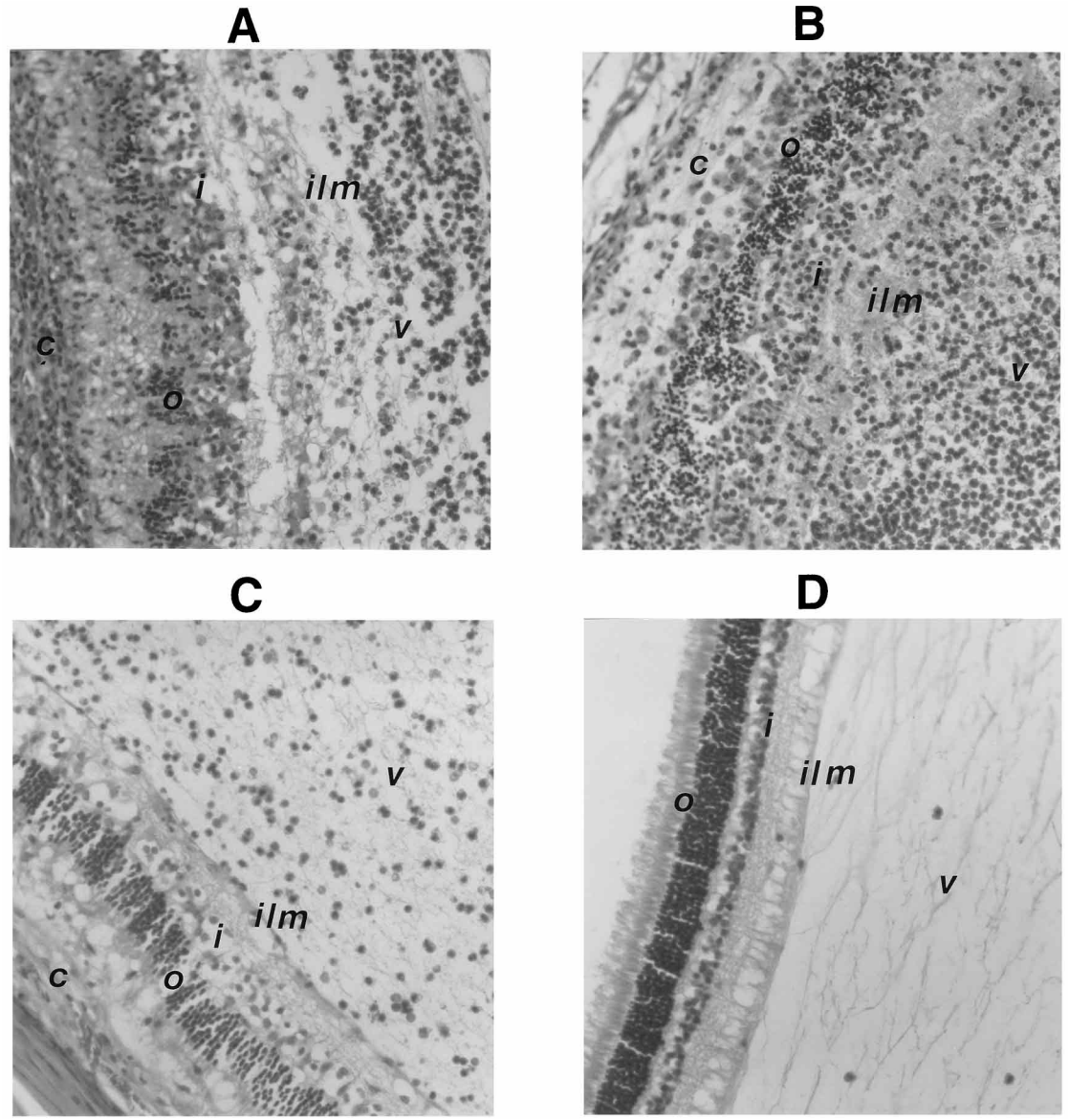

FIG. 3. Hematoxylin-and-eosin-stained sections through *S. aureus*-infected rabbit eyes enucleated 3 days after inoculation. (A) RN6390 (parental strain); (B) ALC136 (*sar* mutant); (C) RN6911 (*agr* mutant); (D) ALC135 (*agr sar* double mutant). v, vitreous; ilm, inner limiting membrane; i, inner nuclear layer; o, outer nuclear layer; c, choroid. Original magnification, ×40.

the Tukey-Kramer honestly significant-difference test was used to make pairwise comparisons between the infection groups. Red reflex was also evaluated. Figure 4 shows the median SLE scores assigned for each infection group. Median SLE scores of 1 or less, with a normal red reflex, were observed for all experimental groups on PID 1. This is consistent with the results of ERG and histopathological analysis, which showed little or no change in B-wave amplitude and essentially normal histology in all infection groups 24 h after inoculation. However, by PID 2, the median SLE score for eyes infected with the parental strain RN6390 increased to 10.5, indicating the onset of a severe inflammatory reaction which involved both the anterior and posterior segments. Furthermore, the red reflex in this infection group was either markedly diminished or absent by PID 2. The median SLE scores on PID 3 (10.0) and PID 5 (9.75) did not change substantially from that observed on PID 2, and a white reflex was apparent in all but one of eight animals on both PID. SLE scores for both ALC136 (*sar* mutant)- and RN6911-infected eyes exhibited a temporal pattern of inflammatory response similar to that of the parental-strain-infected eyes (Fig. 4). However, unlike the parental-strain-infected eyes, 4 of 8 (50%) ALC136-infected eyes and 8 of 11 (72%) RN6911-infected eyes retained some red reflex by PID 5.

A delayed onset of inflammatory response was observed in eyes infected with ALC135 (*agr sar* double mutant) compared to infections with the parental strain, ALC136, or RN6911. On PID 2, the median SLE score in ALC135-infected eyes was substantially lower than that in any other infection group (Fig. 4). However, on PID 3 and 5, similar SLE scores were observed for all infection groups. Statistical analysis showed no significant difference between the SLE scores obtained for the

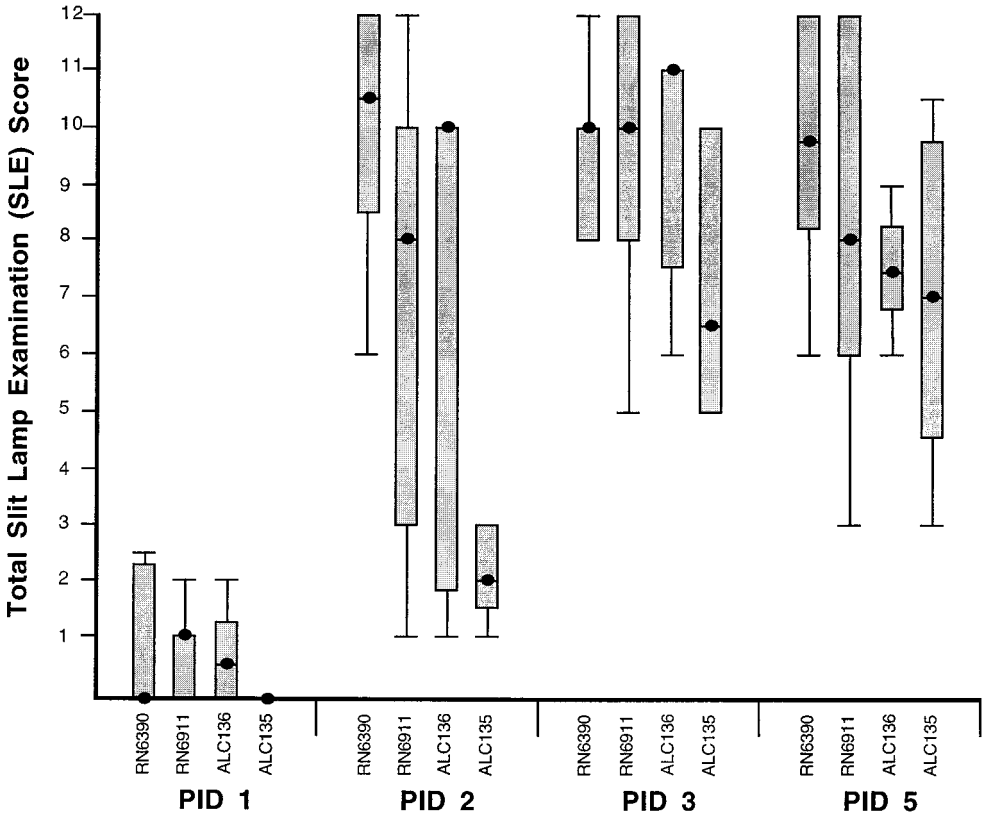

FIG. 4. SLE scores for eyes infected with parental and isogenic *agr* and/or *sar* mutant strains of *S. aureus*. Lines represent the range of scores assigned to each group, 50th percentile (median) scores are shown as dots, and 75th and 25th percentile scores are shown as shaded bars. Strains were RN6390 (n = 8), ALC136 (n = 8), RN6911 (n = 11), and ALC135 (n = 9).

groups on PID 5 (P = 0.218). Even though the SLE scores in ALC135-infected eyes were not significantly different from those in parental-strain-infected eyes on PID 5, none (zero of nine) of the eyes in this infection group exhibited a white reflex, 77% (seven of nine) exhibited a slightly diminished red reflex, and 22% (two of nine) showed a normal red reflex. In contrast, 87% of parental-strain-infected eyes exhibited a white reflex by PID 5.

Global regulatory genes, including *agr* and *sar*, are known to control expression of multiple *S. aureus* virulence factors. Because of the clinical importance of *S. aureus*, these genetic elements have been the subject of considerable interest. The *agr* global regulatory locus consists of two divergent transcriptional units, RNAII and RNAIII. RNAII includes four open reading frames, *agrA*, *-B*, *-C*, and *-D*, all of which are required for *agr* function (3, 15, 19, 27). Sequence analysis suggests that *agrA* and *agrC* comprise a two-component signal transduction system; AgrC, the membrane-bound sensor component, perceives fluctuations in environmental parameters and transmits a signal via a phosphorylation event to AgrA, which then activates transcription of RNAII and subsequently RNAIII. RNAIII then modulates the transcription of exoprotein genes, possibly by an antisense mechanism involving target mRNA (22). Recent reports, however, suggest that the induction and function of the *agr* system involve several additional components. For example, during mid- to late-exponential-phase growth, interaction between *sar* and *agr* is required for optimal RNAII and RNAIII transcription (8, 13). Balaban and Novick (4) have noted the existence of an activating peptide which is secreted after the exponential phase and induces RNAIII transcription. Additionally, the expression of certain exoproteins (e.g., alpha-hemolysin) and not others (e.g., protein A) requires an additional signal that is independent of *agr* and which occurs during post-exponential-phase growth (37). Exoprotein production by *S. aureus* is therefore a complex and carefully coordinated process that involves the interplay of multiple genes, some of which are regulatory in nature. This complexity is reflected by the inability to stably complement *agr* and *sar* global regulatory mutations in *trans* (5a, 14a), which represents a limitation of virulence studies.

The relative roles of *agr* and *sar* in regulating the virulence of *S. aureus* in endophthalmitis were assessed in the present study. Intravitreal injection of as few as 10 to 100 CFU of wild-type *S. aureus* was shown in this and previous studies to lead to massive infiltration of inflammatory cells into the anterior and posterior segments of the eye, severe retinal necrosis, and almost complete loss of neuroretinal responsiveness within 3 days of inoculation (5). Partial attenuation of this fulminant disease course was observed in experimental endophthalmitis caused by an *agr* mutant strain, suggesting that, at least in part, toxin production by *S. aureus* contributes to the severity of infection (5). In the present study, it was found that an *agr sar* double mutant, ALC135, caused no disruption of retinal architecture and, with respect to neuroretinal responsiveness, was indistinguishable from uninfected surgical controls at the times tested. The double mutant was also significantly less virulent than either of the mutant strains harboring single mutations, ALC136 (*sar* mutant) and RN6911 (*agr* mutant). In vitro phenotypic analysis of RN6390, RN6911, ALC136, and ALC135 shows a significant reduction in exopro-

tein expression by the double mutant (ALC135) compared to that by the parental and single mutant strains (6). This suggests that exoproteins expressed under the joint control of the *sar* and *agr* loci play a central role in the pathogenesis of endophthalmitis. It is also noteworthy that even though the double mutant induced a moderate level of inflammatory response in rabbit eyes, as determined by SLE and histological analysis, little tissue damage or loss of retinal function was associated with the infection. This suggests that the inflammatory response to the presence of a nontoxigenic *S. aureus* mutant strain such as ALC135 does not make a major contribution to pathogenesis, at least within the first 5 days of infection.

The observation of a partial attenuation in the virulence of *agr* mutant strain RN6911 in experimental endophthalmitis confirms the results of previous studies using different host strains (ISP479 [wild type] and ISP546 [*agr* mutant]) (5). However, in the present study, the *agr* mutant strain was significantly more virulent than a strain harboring mutations in both the *agr* and *sar* loci (ALC135). The mechanism underlying this intermediate level of virulence is not clear. It is possible that a functional *sar* locus in an *agr* mutant background continues to activate virulence traits at a reduced level (e.g., beta-hemolysin [6, 9]), resulting in only partial attenuation in virulence. Alternatively, it is possible that multiple virulence traits are involved in the pathogenesis of *S. aureus* endophthalmitis and that only some of these are attenuated in the *agr sar*$^+$ strain. The combined effect, however, of insertional mutations in both *agr* and *sar* leads to near-complete attenuation of virulence traits that result in the vision-compromising tissue damage in endophthalmitis.

It is of interest that no significant difference was observed in the rates or extents of B-wave amplitude reduction between the *sar* mutant strain (ALC136)- and parental-strain (RN6390)-infected eyes. This contrasts with the findings of Cheung et al. (6), who observed that ALC136 was attenuated in virulence compared to RN6390 in a rabbit model of endocarditis. That such model-specific differences should occur is not necessarily surprising, given the considerable difference in microenvironmental conditions encountered by the bacterium at these two infection sites. Nonetheless, the wild-type levels of virulence associated with ALC136 in the present study were unexpected since a number of overlaps exist between the *agr* mutant strain and the *sar* mutant strain with respect to in vitro exoprotein production (6, 9). One important difference between these mutants noted previously is the overexpression of both proteolytic and lipolytic activities by *sar* mutant strains, which may contribute to the virulence of ALC136 observed in experimental endophthalmitis (7, 13). In the present endophthalmitis model, a lipase-deficient derivative of ALC136 (ALC599; *geh* null mutant [unpublished data]) was tested, but no attenuation in virulence was found (data not shown). This rules out the involvement of elevated levels of lipase in the virulence of strain ALC136; however, the elevated proteolytic activity expressed by the *sar* mutant may contribute to the virulence observed. Whether proteolytic activity also contributes to the virulence of parental strain RN6390 cannot be concluded from this study.

Many pathogenic bacteria possess the ability to modulate the expression of multiple genes in response to microenvironmental changes, presumably to enhance survival under varying conditions (4, 11, 19). Virulence gene expression is an integral part of this response. For the pathogen, virulence gene products may exploit environmental sources of nutrition. The consequences for the host, however, are often severe tissue damage or even death. Toxin damage within the eye is particularly devastating due to the nonregenerative nature of photoreceptors and neuronal cells lining the posterior segment. Direct destruction of these tissues by extracellular toxins appears, from the results of this study, to explain the high rate of vision loss that occurs in endophthalmitis caused by *S. aureus*, despite the aggressive therapeutic measures currently in use. By identifying the key virulence factors, from among the *agr*- and *sar*-regulated exoproteins, that contribute to the pathogenesis of endophthalmitis, new information-based therapeutic approaches may be developed to improve visual outcome.

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