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Allele-Dependent Differences in Quorum-Sensing Dynamics Result in Variant Expression of Virulence Genes in *Staphylococcus aureus*

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Agr is an autoinducing, quorum-sensing system that functions in many Gram-positive species and is best characterized in the pathogen *Staphylococcus aureus*, in which it is a global regulator of virulence gene expression. Allelic variations in the *agr* genes have resulted in the emergence of four quorum-sensing specificity groups in *S. aureus*, which correlate with different strain pathotypes. The basis for these predilections is unclear but is hypothesized to involve the phenomenon of quorum-sensing interference between strains of different *agr* groups, which may drive *S. aureus* strain isolation and divergence. Whether properties intrinsic to each *agr* allele directly influence virulence phenotypes within *S. aureus* is unknown. In this study, we examined group-specific differences in *agr* autoinduction and virulence gene regulation by utilizing congenic strains, each harboring a unique *S. aureus agr* allele, enabling a dissection of *agr* locus-dependent versus genotype-dependent effects on quorum-sensing dynamics and virulence factor production. Employing a reporter fusion to the principal *agr* promoter, P3, we observed allele-dependent differences in the timing and magnitude of *agr* activation. These differences were mediated by polymorphisms within the *agrBDCA* genes and translated to significant variations in the expression of a key transcriptional regulator, Rot, and of several important exoproteins and surface factors involved in pathogenesis. This work uncovers the contribution of divergent quorum-sensing alleles to variant expression of virulence determinants within a bacterial species.

Bacterial pathogenicity results from a complex interplay of virulence factors with regulatory systems that respond to multiple external signals from the host environment and the bacterial population. Polymorphisms within critical regulatory genes are increasingly recognized within members of the same species and are thought to contribute to strain-specific differences in disease outcomes (13, 57). In the major human pathogen *Staphylococcus aureus*, virulence gene expression is controlled by several interacting regulatory systems, including two-component modules, alternative sigma factors, and transcription factors (53). Many of these regulatory genes are observed to vary across strains, often associated with profound effects on the virulon (2, 8, 12, 40).

A key multiallelic regulatory system in *S. aureus* that influences the overall regulatory network and the virulon is the accessory gene regulator (*agr*) quorum-sensing system. *agr* is an autoinducing, two-component system that reciprocally controls surface factors and secreted enzymes and cytotoxins in coordination with changes in the concentration of a secreted pheromone (54) and is critical for pathogenesis in many contexts (1, 17, 25, 75). *agr* encodes the autoinducing peptide (AIP) pheromone (known as AgrD in its proform), its processor, AgrB, and its detector, the AgrC receptor histidine kinase. At a critical AIP concentration, which may mirror a threshold population density, or quorum, AgrC activation leads to phosphorylation of its response regulator, AgrA, and autoinduction of the *agr* promoters P2 and P3. P3 activation results in expression of the regulatory RNA, RNAIII, which affects the expression of a plethora of virulence genes both directly, by binding to their transcripts (31, 56), and indirectly, by acting on intermediary transcription factors such as Rot (10, 22, 65). Additional genes implicated in virulence are regulated by *agr* independently of RNAIII in an AgrA-dependent manner (62). Expression of *agr* is influenced by other regulatory factors (8, 28, 46–48, 68, 80), indicating that quorum-sensing inputs are integrated with additional cellular signals in the control of the bacterial virulon.

The *agr* locus has diverged widely among strains of *S. aureus*, with polymorphisms centering on *agrB*, *agrD*, and *agrC* (see Fig. S1 in the supplemental material), genes whose products interact molecularly. This diversity has resulted in four *S. aureus agr* pherotypes or specificity groups, which are referred to as groups I through IV (33, 35) (the corresponding *agr* alleles, which here denote the entire group of genes comprising the *agr* locus, are, respectively, referred to as *agr*-I through -IV). The specificity groups have the interesting functional property of heterologous mutual inhibition; that is, the unique AIP of a given group will generally cross-inhibit the *agr* signal in strains belonging to a heterologous group (35). Diversity in the *agr* locus extends far beyond *S. aureus*: all other staphylococcal species examined to date possess a unique *agr* locus with one or more allelic variants (20, 35, 37, 59), several of which are known to mediate heterologous interference with *S. aureus agr* (24, 37, 58; J. S. Wright and R. P. Novick, unpublished data), and *agr* homologs have been identified in many other Gram-positive bacterial genera (4, 19, 51, 54, 67, 78). *agr* divergence and cross-interference may therefore be a wide-ranging phenomenon among the staphylococci and related organisms.

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Interestingly, *S. aureus agr* specificity groups appear to correlate with different infection types. For example, strains belonging to *agr* group III are the primary cause of menstrual toxic shock syndrome (TSS) (34, 35) and are responsible for a large subset of Panton-Valentine leukocidin (PVL)-associated necrotizing pneumonia cases (26, 70), while most strains producing the toxin responsible for staphylococcal scalded skin syndrome, exfoliatin, belong to *agr* group IV (33, 50). These associations appear to involve the acquisition by strains in each group of particular mobile genetic elements (MGEs) carrying toxins (77); however, other transmissible elements and virulence genes correlate poorly with *agr* alleles (34, 77). The molecular basis underlying the predilections of *agr* pherotypes for *S. aureus* pathotypes is currently unknown.

At least two not mutually exclusive hypotheses have been proposed to explain the broader biological significance of *agr* diversity. The first hypothesis is that *agr* interference may serve to isolate bacterial populations through niche competition, limiting genetic exchange and promoting evolutionary diversification. In support of this prediction, each *S. aureus agr* allele is observed to be associated with a specific set of overall genotypes (34, 77), pointing to *agr* group differentiation as a primary evolutionary event that preceded genotypic divergence; however, as mentioned above, examples of virulence determinants that strongly correlate with *agr* alleles are limited (34, 77). The second hypothesis is that *agr* diversity results in quorum-sensing systems with variant signaling properties, ultimately affecting the overall virulence gene regulatory network. In support of this hypothesis are previous observations that relate to other groups; induction of RNAIII occurs early (in early exponential phase) in *agr*-IV strains (33) and later (in late exponential phase) in *agr*-III strains (69; our unpublished data). These timing differences are associated with expression of virulence genes at different phases of cell growth (33, 69; A. M. Figueiredo, H. F. Ross, and R. P. Novick, unpublished data). It is unknown to what extent the temporal differences in *agr* activation are due to intrinsic properties particular to each quorum-sensing allele as opposed to extrinsic influences of the overall bacterial regulatory network (which are particular to each strain) on the *agr* promoters and gene products. The direct impact of intrinsically different *agr* signaling dynamics on the expression of the virulon, and by extension on potential strain pathotypes, is similarly unknown.

In this study, we have addressed these questions by analyzing the four divergent *S. aureus agr* alleles in identical genomic contexts. This approach has allowed us to dissect the contribution of each allele, independent of the background genotype, to signal transduction and accessory gene production and begin to test the hypothesis that concerted allelic variants in an autoinducing regulatory system provide the framework for adaptive virulence phenotypes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. aureus* strains used in this study are listed in Table S1 in the supplemental material. RN6734 is a derivative of 8325-4 (52) and is our standard *agr* group I laboratory strain. RN7206 is a derivative of 8325-4, in which the *agr* locus has been replaced by *tetM*. Newman, an *agr* group I prototype and a human clinical isolate, was originally isolated from a secondarily infected tubercular osteomyelitis lesion (21). HF6122 is a Newman derivative carrying the *agr*::*tetM* replacement. RN6607 (also known as 502A), an *agr* group II prototype, was originally isolated from the nasal mucosa of a healthy carrier (66). RN3984 is a prototypical *agr* group III strain and a menstrual toxic shock syndrome isolate (39). RN4850 is a group IV prototype isolated from a patient with staphylococcal scalded skin syndrome (33). Cloning was performed with *Escherichia coli* strain DH5α. All clones were first transformed into RN4220, our standard recipient for *E. coli* DNA, or its derivatives containing a site-specific integrase before transduction to other strains.

S. aureus cells from overnight plates containing the appropriate selective antibiotics (chloramphenicol 10 μg/ml, erythromycin 10 μg/ml, and/or cadmium 0.1 mM) were used as base inocula for all experiments. Subsequent growth in Casamino Acids-yeast extract-glycerophosphate (CYGP) broth without glucose and without antibiotics was performed at 37°C with shaking. Cell density was determined by using a ThermoMax microplate reader (Molecular Devices) to measure the optical density at 650 nm (OD_{650}) of 100 μl samples in 96-well round-bottom assay plates.

Plasmid construction. The plasmids used in this study (see Table S1 in the supplemental material) were constructed by cloning PCR products amplified with oligonucleotide primers obtained from Integrated DNA Technologies (Coralville, IA), as listed in Table S2 in the supplemental material. Clones were sequenced by the Skirball DNA Sequencing Core Facility or Macrogen (Rockville, MD). Plasmid pJC111, which contains a cadmium resistance cassette and the SaPI-I att_S sequence (23), was used as the backbone vector for *agr* locus constructs. *agr* alleles II through IV were amplified from RN6607, RN3984, and RN4850, respectively, and introduced via blunt ligation into the polylinker of pUC18 at the HincII site. *agr*-I was obtained from pJC1000, which contains the *agr* locus cloned from RN6734 in pUC18 (23). Constructs were then subcloned into pJC1111 using restriction enzymes SphI and KpnI. Chimeric *agr* loci were constructed by swapping SphI-BseRI and BseRI-KpnI fragments involving the unique BseRI site in a conserved region immediately upstream of *agrB*. pJC1111 derivatives containing *agr* locus constructs or the vector alone were integrated into the *S. aureus* genome at the SaPI-1 att_C site by electroporating into RN9011, which expresses the SaPI-1 integrase. Chromosomal *agr* constructs within RN9011 derivatives were amplified and resequenced to confirm the absence of adventitious mutations, and multiple independent transductants were utilized in subsequent experiments.

An $agrp_3$ β-lactamase reporter was constructed by moving the $agrp_3$-*blaZ* fragment from pJC1349 (containing $agrp_3$ cloned from RN6734) to pEG813, a suicide vector containing a cloned ϕ11 att_P cassette (amplified from pLL29 [44]). An analogous plasmid lacking a promoter was constructed by moving the promoterless *blaZ* gene from pCN41 to pEG813. These plasmids were individually integrated into the ϕ11 att_P site by electroporating into RN11679, which contains the ϕ11 integrase encoded in pLL2787 (44). pLL29 and pLL2787 were kindly provided by Chia Lee. β-Lactamase fusions to virulence gene promoters were constructed by PCR amplification of the *lukSF-PV* promoter from ϕSLT, *hla* promoter from RN6734, and the *tsst-1* promoter from RN4282 and cloned into pJC1280. A constitutive variant of the β-lactamase promoter was moved from pJC1122 to the multiple-cloning site (MCS) of pCN41.

Reporter assays. Strains containing β-lactamase fusions were transferred from overnight plates into CYGP without glucose, washed once with fresh media to remove carry-over AIP, and then inoculated into CYGP without glucose at OD_{650} of 0.005. Cells were grown at 37°C with shaking to OD_{650} of 0.05 and were diluted with fresh media to OD_{650} of 0.01, representing T_0 and corresponding to approximately 1×10^7 CFU/ml. This dilution step permitted further reduction of residual *agr* and reporter transcripts, cell density normalization, and synchronization in early exponential phase. Samples were collected at the indicated time points and stored at −80°C. Samples were thawed on ice and diluted with media to appropriate densities for turbidity measurements and enzyme assays. Assay of β-lactamase activity was performed by the nitrocefin method as described previously (36). Assay data were normalized to β-lactamase units (V_{max}/OD_{650}).

Exoprotein analysis. For qualitative analysis of hemolysin production, cells were patched on sheep's blood agar (SBA) plates, followed by overnight growth at 37°C. For SDS-polyacrylamide gel electrophoresis

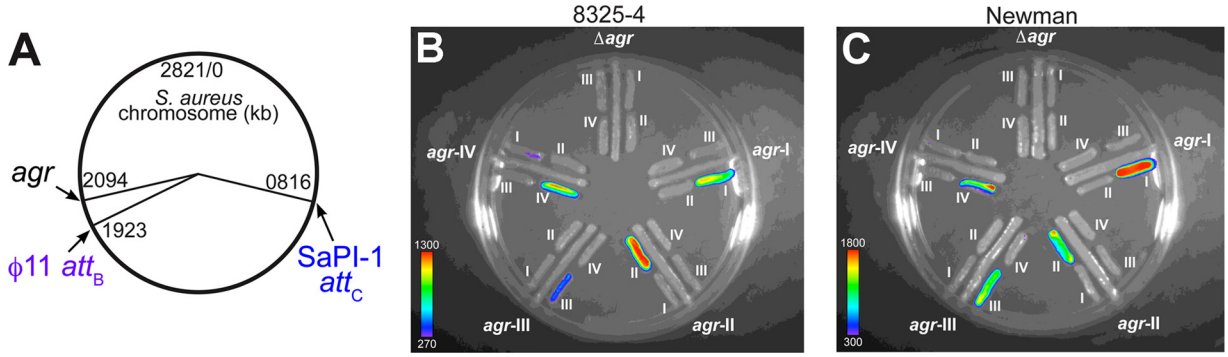

FIG 1 Chromosomal positions of loci under study and bioluminescent typing assay of congenic *S. aureus* strains. (A) Chromosomal positions in NCTC 8325 of the native *agr* locus and SaPI-1 and ϕ11 attachment sites. The ϕ11 att_B site is identical to the integration site of phage NM1 in Newman (5), and the indicated sites are located at analogous distances in the Newman chromosome. (B, C) Bioluminescent plate assay. Congenic strains with the indicated *agr* allele are streaked radially. The reporter strains (RN9688, RN9689, RN9690, and RN9691 [77]), each carrying an allele-specific AgrCA two-component signaling pair plus a $agrp_3$-*lux* fusion, are patched alongside the congenic strains, each of which activates one of the reporters according to the specificity of its AIP. Reporters are identified by roman numerals designating their specificity group. Bioluminescence was detected with a Hamamatsu charge-coupled-device camera and is presented as a pseudocolor image, with the color bar indicating the signal intensity in counts.

(SDS-PAGE) analysis of exoprotein profiles, cells were washed and grown as described for reporter assays, samples were collected at the indicated time points, and cell density was measured. Culture supernatants were isolated by centrifugation, and sample volumes within each time point were normalized on the basis of culture densities. Protein was precipitated with trichloroacetic acid, resuspended in SDS loading buffer, and separated by SDS-PAGE in a 12% polyacrylamide gel, followed by visualization using Coomassie blue dye (41). Secreted β-lactamase from the same samples was analyzed by zymography with a method adapted from the work of others (49, 63). Samples were run on a 15% polyacrylamide gel, and the gel was washed in water twice for 20 min, incubated in 500 ml renaturation buffer (50 mM sodium phosphate buffer [pH 7.0], 1% Triton X-100, 0.1 mM ZnSO4) at 37°C for 4 h, and then overlaid with filter paper presoaked in 50 mM sodium phosphate buffer, pH 7.0, containing the chromogenic substrate nitrocefin at 0.25 mM. Protein identification by mass spectrometry was performed by proteolytic digestion with trypsin of excised gel slices containing the protein band of interest, followed by liquid chromatography-mass spectrometry (LC-MS) (Skirball Protein Analysis Facility).

RESULTS

Construction of congenic strains containing unique *agr* alleles. In order to construct congenic strains, each harboring a unique *agr* allele, we inserted the *agr* genes, cloned from the chromosome of prototypical *S. aureus* strains belonging to each *agr* specificity group (see Table S1 in the supplemental material), in single copy within a Δ*agr* strain. We utilized the staphylococcal pathogenicity island (SaPI)-1 att_C locus (16, 23, 24) as the insertion site, noting that this position is at a roughly similar distance from the chromosomal origin of replication relative to the native *agr* locus (Fig. 1A), enabling similar gene copy numbers at these locations in exponentially growing cells (14, 81). We employed two *S. aureus* strain backgrounds, RN7206 and HF6122, which are Δ*agr* mutant derivatives of NCTC 8325-4 and Newman, respectively (see Table S1). These strains were selected because of their differences in expression of transcriptional regulators, including Sae (2) and σ^B (8), their differences in *agr* activity (8), their global differences in virulence factor production (29), and their wide use by several labs as prototypical wild-type (WT) strains (6, 15, 17, 61, 65). Integration of SaPI-1 into its att_C site invariantly occurs in the same orientation (64), and we verified the identical orientation of each *agr* locus in the chromosome by PCR (see Fig. S2A in the supplemental material). The functionality of the *agr* system from this site and the specificity group identity for each resulting congenic strain were confirmed using the plate-based *agr* typing assay (77) (Fig. 1B and C).

Cell growth in culture was observed to be identical for congenic strains of the Newman background (see Fig. S2C in the supplemental material) and nearly identical for those of the 8325-4 background (see Fig. S2B); the *agr*-III and Δ*agr* strains in this background were associated with slightly increased culture turbidity over time, consistent with earlier observations with *agr* mutant cells (our unpublished data). These differences were eliminated when early exponential-phase cells were diluted to a lower cell density (see Fig. S2B, inset), presumably because of dilution of residual *agr* transcripts and AIP carried over during subculture.

Allelic differences in *agr* induction dynamics. We have previously observed differences in the timing of RNAIII transcription, the immediate consequence of *agr* autoinduction, across divergent *S. aureus* strains belonging to different *agr* groups; most notably, RNAIII is induced very early during growth in group IV isolates, about 3 h earlier than in other groups during standard culture conditions (33), and group III strains have been observed to induce RNAIII transcription late compared to the other groups (69; our unpublished data). The contribution of the intrinsic properties of each *agr* allele in determining the timing of RNAIII expression, relative to the effects of extrinsic regulatory factors elsewhere in the genome, is not known. Similarly, a uniform analysis of group-specific differences in *agr* activation magnitude has not been reported. In order to explore the activation timing and maximal signal strength of each *agr* allele in the congenic strains, we employed a fusion of the β-lactamase reporter gene to the principal *agr* promoter, P3. The $agrp_3$-*blaZ* construct was cloned into a suicide plasmid containing the phage ϕ11 att_P sequence (42) and inserted in single copy into the corresponding chromosomal att_B site (Fig. 1A). A promoterless *blaZ* construct at this position resulted in reporter activity barely detectable over background (see Fig. S3A in the supplemental material), indicating that gene expression from this chromosomal site is not affected by exogenous transcriptional read-through.

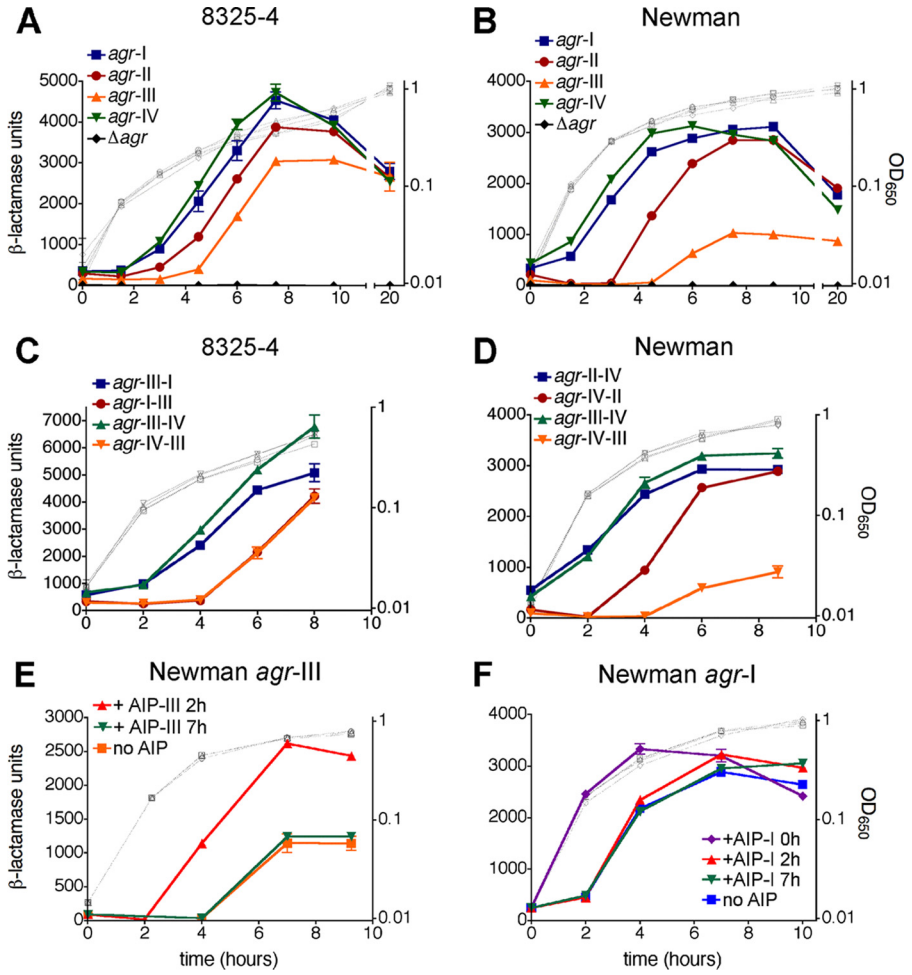

FIG 2 Tests of P3 promoter activity in congenic strains containing unique *agr* alleles. *S. aureus* cells of the 8325-4 (A, C) or Newman (B, D) background containing the indicated *agr* allele (at SaPI-1 att_C) and a chromosomal $agrp_3$-*blaZ* transcriptional fusion (ϕ11 att_B::pEG835) were assayed for β-lactamase activity during growth in triplicate cultures. (C, D) *agr-x-y* refers to chimeric *agr* loci, with *x* representing the 5′ fragment containing RNAIII and the intergenic region, and *y* representing the 3′ fragment containing *agrBDCA* (see Fig. S1 in the supplemental material). (E, F) Effect of exogenous AIP on $agrp_3$ activity. *S. aureus* cells derived from strain Newman containing *agr*-III (E) or *agr*-I (F) and the $agrp_3$-*blaZ* reporter were assayed before or after addition at the indicated time points of AIP at 1 μM. Assay data are presented as β-lactamase units ± standard errors of the means (SEM) (closed symbols, left axis) and growth as optical density measurements (gray open symbols, right axis).

Using the $agrp_3$-*blaZ* reporter, we next determined whether the chromosomal position of the *agr* locus affected its activation. We observed similar $agrp_3$ induction kinetics and maximal signal levels during growth in culture in cells containing the *agr*-I locus at its native site or at the SaPI-1 att_C site (see Fig. S3B in the supplemental material). We also observed similar exoprotein production levels, as measured by SDS-PAGE of culture supernatants (see Fig. S4 in the supplemental material) and SBA analysis (see Fig. 3B, top row), with *agr* in its native site or at the SaPI-1 site within the same genomic background. These results indicate that the SaPI-1 att_C site functionally substitutes for the native *agr* site for these studies.

We next used the $agrp_3$-*blaZ* reporter to analyze *agr* activation by the congenic strains containing unique *agr* alleles. The timing and maximal activity of reporter induction varied for both 8325-4 and Newman backgrounds and was significantly dependent on the particular *agr* allele (Fig. 2). With the 8325-4 strains harboring *agr*-IV and -I, reporter activity was induced with the earliest kinetics, with 50% maximal activities reached at approximately 4.4 and 4.7 h, respectively (Fig. 2A). This was followed by *agr*-II and -III cells at approximately 5.3 and 5.8 h, respectively (Fig. 2A). With the Newman strains, a similar ordering in allele-specific induction timing was observed (Fig. 2B): *agr*-IV cells displayed the earliest induction kinetics, followed in order by *agr*-I, -II, and -III (Fig. 2B; 50% maximal activities reached at approximately 2.4, 2.9, 4.6, and 5.6 h, respectively). Overall, with respect to maximal activation levels, these were higher for the 8325-4 strains compared to the Newman strains, consistent with previous reports with the wild-type strains (8, 27). With both congenic strain sets, the greatest induction levels were observed with *agr*-I and *agr*-IV and the lowest with *agr*-III (Fig. 2). Reporter induction by the *agr*-III cells was particularly low in the Newman background (Fig. 2B).

To determine whether the observed allele-dependent variations in reporter activity are due specifically to effects on the P3 promoter, as opposed to general effects of the *agr* alleles on cellular transcript levels, protein levels, or protein export, we tested strains expressing the β-lactamase reporter from a promoter known to be

unaffected by *agr*. To this end, we utilized a stable constitutive variant of the *blaZ* promoter (*blaZ*p_c; see Fig. S3C in the supplemental material), the native version of which is known to be *agr* independent (55). In strains carrying either of the two *agr* alleles most variant in induction properties, *agr*-III and -IV, we observed that the reporter signal from *blaZ*p_c was largely unaffected by the *agr* allele present (see Fig. S3D and E). The allelic differences in *agr*p_3 reporter activity are therefore due to specific effects on transcription from this promoter and are not due to general effects on intra- or extracellular reporter protein levels. Together these results demonstrate that the *agr* alleles are characterized intrinsically by different activation dynamics, with significant variations in the timing and maximal strength of the *agr* signal. *agr*-III mediates the most delayed and weakest induction signal, *agr*-IV the earliest, and *agr*-I and -IV the strongest. These differences are consistent with previous observations of RNAIII production by *S. aureus* isolates belonging to different *agr* specificity groups (33, 69; our unpublished data). These experiments also demonstrate that strain genomic background affects both the timing and magnitude of *agr* induction, in agreement with previous reports on the effects of σ^B levels on *agr* activity (30).

The *agrBDCA* region is responsible for variations in induction properties. Intrinsic properties of the *agr* locus that could influence induction dynamics include differences in the rate of AIP processing and secretion, AIP-AgrC interaction kinetics, AgrC phosphorylation and phosphotransfer rates, and *agr* promoter activities. The vast majority of nucleotide polymorphisms among the *agr* alleles occurs in the hypervariable *agrB-agrD-agrC* region (see Fig. S1 in the supplemental material), while a much smaller subset of polymorphisms occurs in RNAIII, the *agr* intergenic region (containing *agr* promoters P2 and P3), and *agrA*, for which the resulting protein sequence is absolutely conserved. Single nucleotide changes in the *agr* intergenic region, however, can have a large impact on expression of this system (71). To determine the relative contribution of each set of polymorphisms to differences in *agr* activation properties, we analyzed *agr* locus chimeras in which locus segments were swapped between alleles at a conserved site immediately upstream of *agrB* (see Fig. S1). These chimeras were functional, as indicated by *agr*p_3 reporter activity (Fig. 2C and D). In each case, the chimeras demonstrated activation signal timing and magnitude that behaved according to the group identity of the fragment containing the P2 transcript genes (*agrBDCA*). For example, in all backgrounds, chimeras involving this region of *agr*-III demonstrated activation kinetics virtually identical to that of the WT *agr*-III allele, and in the Newman background in which chimeras involving *agr*-II were tested, the construct containing the *agr*-II P2 transcript genes behaved similarly to WT *agr*-II (Fig. 2C and D). These results indicate that the P2 open reading frames (ORFs) (*agrBDCA*), as opposed to the P2 and P3 promoters and RNAIII, determine allelic differences in *agr* induction properties.

Effects on *agr* induction by addition of exogenous AIP. The magnitude of induction by the *agr*-III allele is lower than other groups, particularly in the Newman background. In order to determine whether this weak signal was due to a decreased capacity for AgrC-III to activate in response to ligand, we tested whether the signal could be increased by addition of exogenous AIP-III at different time points during the autoinduction process. We added a saturating concentration of AIP-III to Newman cells expressing the *agr*-III allele at a time point before the onset of *agr*p_3 induction (2 h) and after maximal induction levels were reached (7 h) and measured *agr* reporter activity. Addition of AIP-III at 2 h resulted in *agr*p_3 activity that was earlier and of higher magnitude than that observed with no exogenous AIP (Fig. 2E), and the maximal signal level reached was roughly commensurate with that of the other *agr* alleles in this background. Addition of exogenous AIP-III at 7 h, however, did not result in any further increase in *agr* signal strength (Fig. 2E). This result can be interpreted in two ways: either all AgrC receptor molecules are already saturated with endogenous AIP at this late time point or additional AgrC activation does not lead to further transcription from *agr*p_3 or expression of the transcript at this stage of growth, perhaps due to extrinsic, stationary phase-dependent limits on gene expression (32, 38). Activation of *agr*-I was also hastened by adding exogenous AIP-I at the early time point (T_0), but the maximal signal level was not increased (Fig. 2F); further, addition of AIP-I to *agr*-I cells at later time points (2 and 7 h) did not affect induction kinetics or signal strength, consistent with receptor saturation at 2 h and saturation or growth-phase limits at 7 h. These data indicate that the delayed and weakened induction of *agr*-III relative to other alleles is not due to a decreased maximal signaling capacity of AgrC-III and is likely owing, at least in part, to a reduced production of AIP-III, controlled by the products of *agrB* or *agrD*.

Influence of *agr* allele on hemolysin production. Production of exoproteins such as hemolysins in *S. aureus* is controlled by both the *agr* system and by regulatory factors encoded elsewhere in the genome, the expression and activity of which often vary across strains (9, 15). Given the differences in induction timing and magnitude among the *agr* alleles, we hypothesized that these differences would lead to variations in exoprotein production when examined in congenic backgrounds. As an initial test of this hypothesis, we analyzed hemolysin activity qualitatively on sheep's blood agar (SBA) plates. Strains were cross-streaked with RN4220, which produces only β-hemolysin, to facilitate identification of the activities of different types of hemolysins after overnight growth. An interpretation of the resulting hemolytic patterns is shown in Fig. 3A. As seen in Fig. 3B, the parental strains (the *agr* specificity group prototypes from which each allele was cloned) each produced a unique hemolytic pattern on SBA. The *agr*-III prototype, a toxic shock syndrome (TSS) isolate, produced very low hemolysin levels, characteristic of strains producing the TSST-1 toxin, which is associated with repression of exoproteins (53); in contrast, the *agr*-IV prototype produced large amounts of hemolysins that synergize with β. The global differences across specificity groups were no longer apparent with the congenic strains (Fig. 3B), illustrating the role of background genotype on hemolysin activity. Reproducible decreases in the production of β-synergizing hemolysins, however, were observed with the *agr*-III strains compared to their congenic counterparts, particularly in the Newman background; likewise, the *agr*-IV strains appear to produce more β-synergizing hemolysins relative to their congenic strains. β-Synergistic hemolysis on SBA has traditionally been attributed to δ-hemolysin; it is noted, however, that this toxin belongs to the family of phenol-soluble modulins (PSMs) (72), additional members of which can also contribute to the β-synergistic hemolysis phenotype (18) and are also *agr* regulated (72). This contribution may be more relevant in Newman versus 8325-4, because of the very low levels of many non-δ-toxin PSMs in the latter strain background (18). These experiments demonstrate that while differences in hemolysin production in strains with a functional *agr* system are globally deter-

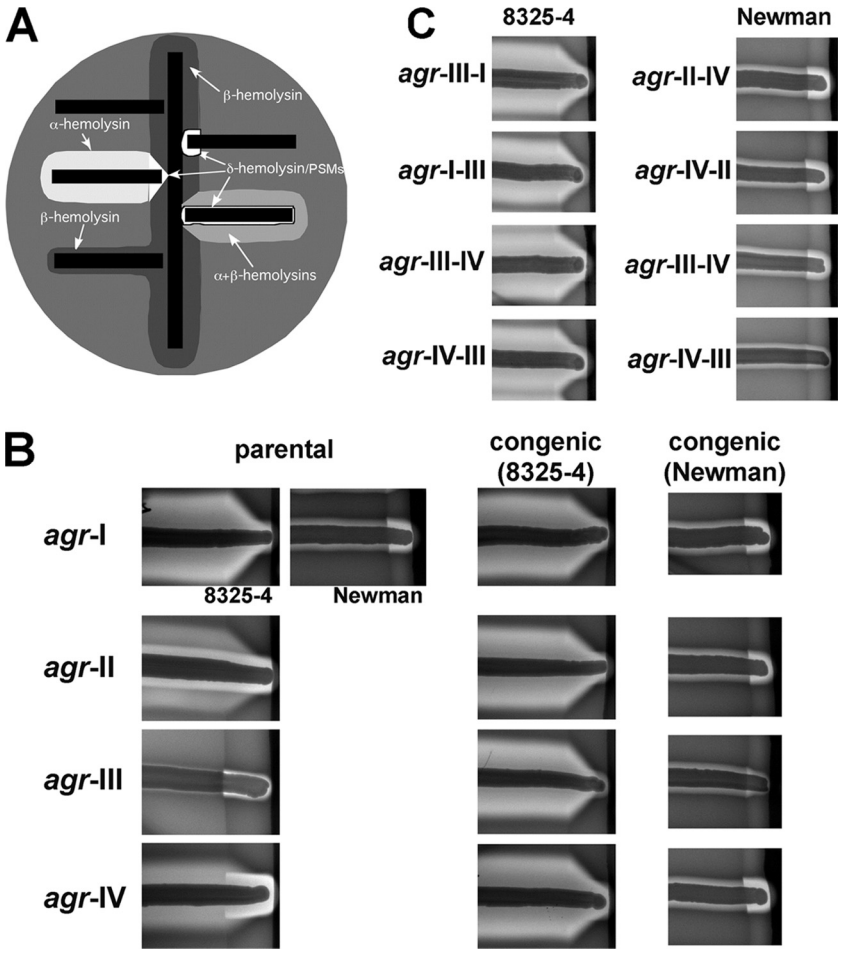

FIG 3 Analysis of hemolytic activity of *S. aureus* strains on sheep's blood agar (SBA). (A) Schematic of hemolytic activities on SBA. Tested bacteria (horizontal black bars) are streaked at a right angle to RN4220 (vertical black bar), a β-hemolysin producer, and the plate is incubated overnight. β-Hemolysin forms a turbid zone of hemolysis; β is synergistic with δ-hemolysin and other PSMs, producing an amplified zone of clearing where they intersect; β inhibits α-hemolysin, producing the V-shaped zone of clearance where they intersect. (B, C) Hemolytic activities on SBA for parental and congenic strains with the indicated *agr* allele cross-streaked with RN4220. Images from each column are from the same plate. Parental strains: *agr*-II, RN6607; *agr*-III, RN3964; *agr*-IV, RN4850. The pattern seen with native *agr*-II is due to the production of β-hemolysin, as well as α and δ and/or other PSMs; Newman has a prophage in the β-hemolysin gene. The α-β mutual inhibition is seen only with Newman *agr*-III because of the low δ-hemolysin and/or other PSM production by group III. In panel C, the chimeras tested correspond to those tested for reporter activity in Fig. 2C and D.

mined by the background genotype, allelic variation of the *agr* locus has an effect on the production of at least one hemolysin type, the β-synergizing hemolysins.

SBA tests of *S. aureus* congenic strains containing the interallele *agr* chimeras tested in Fig. 2 agreed with the corresponding reporter data: chimeras involving the group III *agrBDCA* region generally demonstrated reduced hemolysis, predominantly due to a reduction in β-synergizing hemolysin activity, comparable to the hemolysin pattern observed with strains containing a WT *agr*-III allele (Fig. 3C). The delayed and weakened induction signal mediated by the group III *agrBDCA* region thus results in decreased hemolysin production.

Influence of *agr* allele on exoprotein profiles. To explore the temporal effects of *agr* alleles on production of a range of exoproteins, we analyzed, by SDS-PAGE, exoprotein levels in culture supernatants collected at multiple time points during autoinduction. For these experiments, we focused on the two *agr* alleles with the most widely variant induction properties, *agr*-III and *agr*-IV, and we employed strains carrying the *blaZ*p_c-*blaZ* construct (see Fig. S3 in the supplemental material), which enabled detection, via zymography, of secreted β-lactamase as an *agr*-independent internal control. Over the time points analyzed, β-lactamase levels were similar between *agr*-III and *agr*-IV samples (Fig. 4B and D), with the exception of one sample (Fig. 4B, T20 section, lane IV), for which the corresponding cells had presumably lost the reporter plasmid after overnight growth without selection. In contrast, overall exoprotein profiles, as revealed by Coomassie staining of the same samples, were visibly different across all time points between the two alleles in both 8325-4 and Newman backgrounds (Fig. 4A and C). With respect to overall exoproteins produced, *agr*-III cells of the 8325-4 background appeared to lag behind the congenic *agr*-IV cells by about 2 h, consistent with the temporal gap in *agr*p_3 reporter induction between these strains (Fig. 2A), resulting in differences in exoprotein patterns at each time point that were quite dramatic (Fig. 4A). The differences in exoprotein production between the two alleles was less dramatic

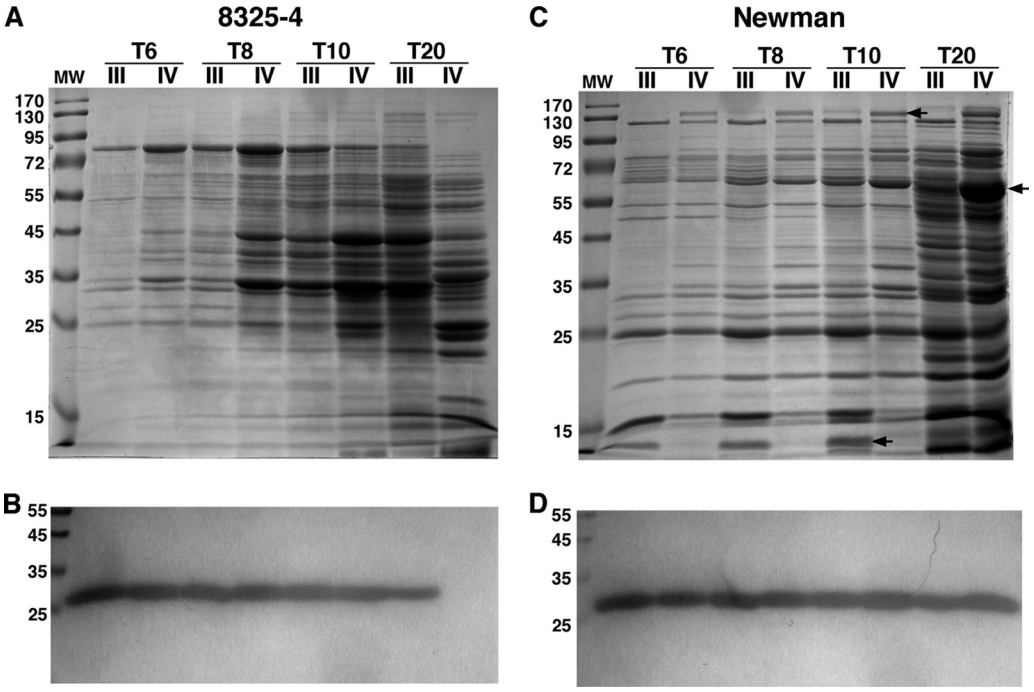

FIG 4 Exoprotein analysis. Culture supernatants were collected at the indicated time points, and exoproteins were isolated and separated via SDS-PAGE. Cells contained the indicated *agr* allele and the *blaZ*p$_c$-*blaZ* construct (pEG832). (A, C) Coomassie staining. Bands indicated by arrows were analyzed by LC-MS. (B, D) Results of β-lactamase zymogram analysis performed after renaturing SDS-PAGE with the chromogenic substrate nitrocefin.

with the Newman strains and, unlike the 8325-4 background, these differences did not appear to reflect a temporal shift between the two *agr* groups. Several distinct bands appeared in *agr*-IV supernatants that were much less abundant in that of *agr*-III, as well as one band present in *agr*-III samples that was not visible in *agr*-IV samples (Fig. 4C). We determined the identity of three of these bands by mass spectrometry. The band at approximately 140 kDa was identified as bifunctional autolysin precursor (Atl), the band at approximately 60 kDa was identified as catalase (KatA), and the band at approximately 12 kDa, which was less abundant in *agr*-IV cells, was identified as fibrinogen binding protein (Fib/Efb). Extracellular fibrinogen binding protein from strain Newman has a molecular mass of 19 kDa, so the band we analyzed may represent a degradation product, as observed in previous studies (29, 43); the differential presence of this band may thus represent differential Efb levels or differential exoproteolytic activities associated with the two strains. These results demonstrate that the *agr* allele can have multiple effects on exoprotein expression in *S. aureus*.

Influence of *agr* allele on regulation of virulence genes. *agr* affects exoprotein expression to a large extent through intermediary transcription factors (53), including the global regulator Rot, the translation of which is blocked by the *agr* effector, RNAIII (10, 22). We reasoned that allelic variation in *agr* activity could result in altered regulation of Rot translation, which may contribute to the observed differences in exoprotein production. To test this prediction, we employed a translational *rot-blaZ* fusion. In previous studies, we observed that the activity of this reporter is decreased in *agr*$^+$ relative to Δ*agr* mutant cells, whereas the reverse is true for the corresponding transcriptional *rot*p-*blaZ* fusion, indicating translational inhibition of Rot by *agr* superimposed on Rot-mediated transcriptional autorepression (22). Here, in both strain backgrounds, we again observed decreased reporter activity in *agr*$^+$ cells relative to Δ*agr* mutant cells (Fig. 5). Notably, the degree of this relative decrease depends on the *agr* allele present and is commensurate with the timing and magnitude of *agr*p$_3$ activation by each allele (see Fig. 2). For both strain backgrounds, maximal inhibition of Rot translation is observed with *agr*-I and -IV, the two alleles with the earliest and strongest *agr* activation dynamics. This is followed by *agr*-II, with an intermediate degree of inhibition (which is also delayed until after 3 h in the Newman strain), and *agr*-III, which demonstrates delayed inhibition of Rot translation in the 8325-4 and very low-level inhibition in the Newman context (Fig. 5).

We next examined the influence of each allele on the temporal activity of several virulence gene promoters that are up- or down-regulated by *agr*, including those for virulence genes encoded in the chromosome (*spa* and *hla*) and encoded by MGEs (*lukSF-PV* and *tst*). As shown in Fig. 6, transcription from these promoters was affected in an allele-dependent manner. In the 8325-4 background, promoter regulation followed the trend previously observed with *agr*p$_3$ activity and Rot inhibition: relative to Δ*agr* mutant cells, cells expressing *agr*-I and -IV demonstrated the greatest temporal change in promoter activity, followed in order by *agr*-II and *agr*-III (Fig. 6A, C, E, and G). In the Newman background, this trend generally applied for *spa*p and *tst*p reporter constructs (Fig. 6B and H), although *tst*p was only minimally induced in *agr*$^+$ strains relative to the Δ*agr* mutant (Fig. 6H). As expected, the *hla* promoter was much more weakly active in the Newman background compared to 8325-4, and interestingly, the activities with each *agr* allele (Fig. 6D) did not follow the trend observed with reporters for *agr*p$_3$, Rot, and other virulence factor promoters. Finally, although the PVL promoter was very strongly activated by

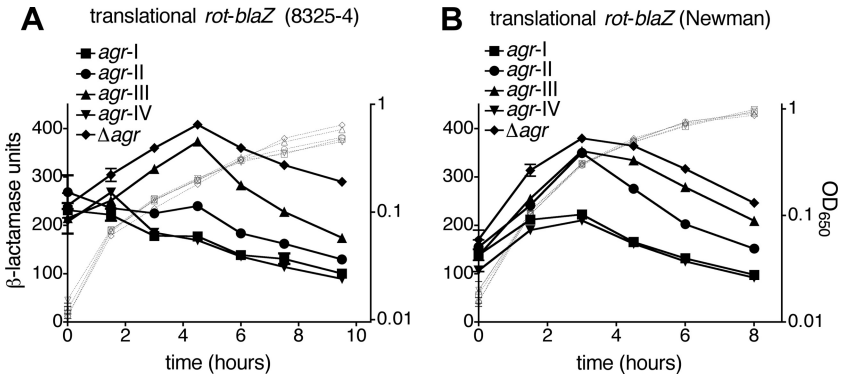

FIG 5 Effect of *agr* allele on inhibition of Rot translation. *S. aureus* cells of the 8325-4 (A) or Newman (B) background containing the indicated *agr* allele and a *rot-blaZ* translational fusion (pRN9162) were assayed for β-lactamase activity. Assay data are presented as in Fig. 2.

agr in 8325-4 strains, its activity was weak in Newman derivatives and either decreased or plateaued after a short period of induction (Fig. 6F); nonetheless, this range of activity depended on the specific *agr* allele, in agreement with the pattern of expression associated with the $agrP_3$ and Rot reporters (Fig. 2B and 5B). These results are consistent with previous findings that PVL expression is dependent on *agr* and is affected by the host background (12, 74). Together these data demonstrate that allelic differences in quorum-sensing signal timing and intensity result in significant variations in the regulation of *agr*-dependent virulence genes.

DISCUSSION

In this study, we have probed the differences in quorum-sensing dynamics by the four *S. aureus agr* alleles expressed by congenic strains. We found significant intrinsic variation in the timing and magnitude of the signal generated from the principal *agr* promoter, P3, indicating that each of the divergent quorum-sensing alleles responds with a unique cell density set point. These signaling variations had consequences for the overall regulatory network and the virulon in *S. aureus*, as they resulted in altered expression of the *agr*-targeted global regulator, *rot*, and several important virulence factors. A hierarchical order in induction timing and strength can be established for the four *S. aureus agr* alleles based on these results, with *agr*-IV and -I the earliest and strongest, followed in order by *agr*-II and *agr*-III. Knowledge of these signaling differences will aid comparative analyses of virulence gene regulation between diverse *S. aureus* isolates.

The differences in *agr* signal generation among the alleles were not due to polymorphisms in the *agr* promoters themselves or RNAIII and instead relied on the *agrBDCA* genes, which contain the region of hypervariability. Aside from the four *agr* ORFs in this segment, we note that an uncharacterized weak promoter, P1, is also present, located between the 5′-most RsaI site in *agrC* and the PvuII site within *agrA*, based on a previous gene fusion study (60); this promoter is believed to function as a constitutive, basal source of *agrA* transcription. A computationally predicted promoter within a conserved region of *agrC* (see Fig. S1 in the supplemental material) may represent the P1 promoter, although this has yet to be tested experimentally. No polymorphism specific to *agr*-II or *agr*-III exists within this predicted promoter segment, so it is unlikely that this DNA region is responsible for the delayed kinetics observed with these two alleles. Because the *agrA* polymorphisms across the four alleles do not result in changes at the amino acid level (the AgrA polypeptide sequence is 100% conserved), the most likely conclusion is that differences in the activities and/or interactions among the hypervariable proteins AgrB, AgrD, and AgrC form the molecular basis of the observed allelic differences in *agr* signaling. Because addition of AIP at an early time point can bypass the delayed and weakened induction characteristic of *agr*-III cells (Fig. 2E), a logical conclusion is that the *agr*-III phenotype is owing at least in part to a reduced production of AIP (controlled presumably by allelic variations in the *agrB* or *agrD* genes) or possibly to its accelerated degradation. Biochemical quantitation of secreted AIP levels determined by each allele over time would address this prediction. If differences in secreted AIP levels are not apparent, a remaining potential source of altered induction properties among the alleles would be variability in cognate AIP-AgrC interaction kinetics. Such differences may be difficult to reveal, however, with the indirect reporter methods currently used (23, 24, 45, 76). Future studies employing purified and reconstituted AgrC will enable quantitative determination of allelic differences in ligand binding and receptor phosphorylation kinetics among cognate ligand-receptor pairs.

In general, different quorum-sensing dynamics resulted in analogous differences in the timing and level of virulence gene production, with interesting features dependent on the host background. With the 8325-4 strain containing the *agr*-III allele, overall exoproteins followed a temporal delay in expression compared to the congenic *agr*-IV strain, and the activities of the four analyzed virulence gene promoters varied according to the activity of the *agr* allele in each strain. In the Newman background, differences in overall exoproteins did not appear to reflect a temporal shift between the alleles analyzed, and allelic differences in transcription from the *hla* promoter did not correlate with *agr* activities. A caveat of the latter experiment is that the maximal agr^+/Δ*agr* mutant difference in *hla*p activity was much lower for the Newman background (approximately 3-fold) compared to the 8325-4 background (approximately 50-fold). In the case of the *spa* and *lukSF-PV* promoters in the Newman background, activities with the *agr* alleles displaying the most delayed kinetics, *agr*-III and *agr*-II, were virtually indistinguishable from that of the Δ*agr* strain, while these two alleles behaved quite differently from the Δ*agr* mutant in the 8325-4 background. The impact of *agr* allele-dependent differences in virulence factor expression thus depends significantly on the background genotype, which may affect expression of *agr*, virulence genes, or both. The 8325-4 and Newman

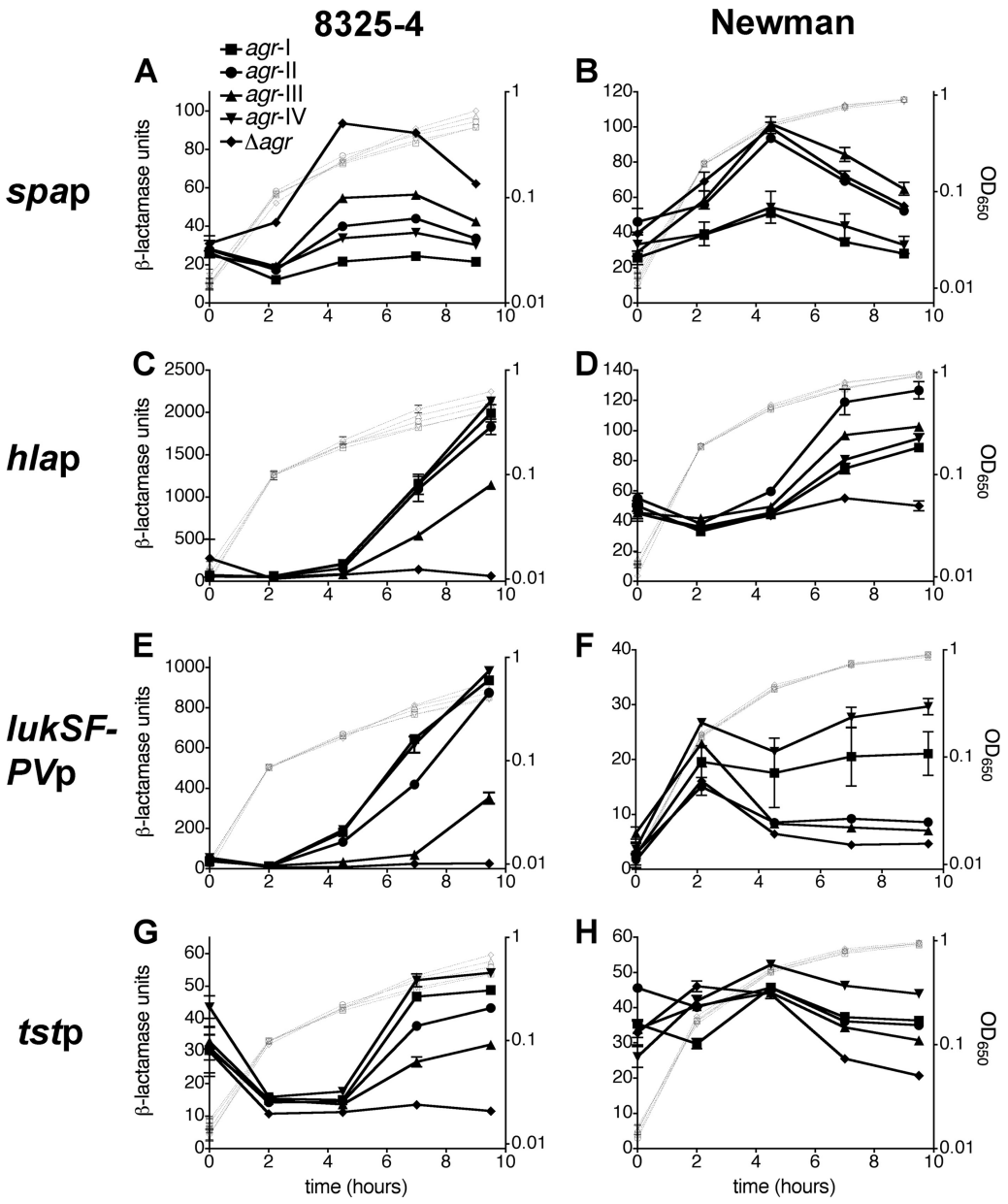

FIG 6 Effects of *agr* allele on virulence gene promoters. *S. aureus* cells of the 8325-4 (A, C, E, G) or Newman (B, D, F, H) background containing the indicated *agr* allele and transcriptional fusion of the *spa* (A, B), *hla* (C, D), *lukSF-PV* (E, F), or *tst* (G, H) promoters to the *blaZ* reporter were tested for β-lactamase activity. Assay data are presented as in Fig. 2.

strain chromosomes are known to differ in at least three areas that influence the virulon and/or pathogenesis: expression of σ^B (8), expression of Sae (2), and presence of prophages (5). Our findings that *agr* and certain of its upregulated targets are expressed at lower levels in Newman compared to 8325-4 are consistent with previous studies on σ^B, an *agr* downregulator whose activity is greatly reduced in 8325-4 (8, 30). The precise extent to which other strain-specific factors contribute to variations in *agr* expression remains to be determined. Nonetheless, these observations underscore the complex interactions of quorum sensing with the overall strain regulatory network.

We examined the variable expression of chromosomal and MGE-encoded virulence genes with the hypothesis that, if expression of horizontally acquired, *agr*-regulated virulence genes varies with the activity of the resident *agr* allele, certain alleles may determine an adaptive virulence phenotype due to a favorable combination of virulence and regulatory elements. With promoters of the *lukSF-PV* and *tst* genes, encoded by phage and SaPI, respectively, we indeed observed a range of activities that depended on the *agr* allele, and the activities and allele-dependent patterns of variability differed significantly between the two host backgrounds. In both backgrounds, expression was the weakest with *agr*-III, the allele that is most commonly represented in strains containing either the phage-encoded PVL toxin or the SaPI-encoded TSST-1 toxin. It is possible that temporal differences in expression of these toxins are beneficial to the organism in partic-

ular contexts, although this remains speculative. We note that our experiments involved reporter fusions to the toxin gene promoters in the absence of their native MGE, which may have affected the degree to which their expression varies with each *agr* allele.

While the data presented here demonstrate that divergent quorum-sensing alleles translate to variant regulation of virulence genes, a key area of future experimentation will involve examining the effects of the *agr* alleles on pathogenesis using animal models of infection. In addition, because the current model for *S. aureus* biofilm development involves localized, *agr*-mediated cellular dispersal from biofilm surfaces (11, 79), temporal differences in quorum-sensing activation may have profound effects on biofilm dynamics. Examining the congenic strains in host infection and biofilm models will enable further tests of the hypothesis that variant quorum-sensing dynamics may constitute a determinant of adaptive virulence phenotypes. Previous observations indicate that the 8325-4 and Newman strain backgrounds differ in their virulence phenotypes in the mouse sepsis model (29) and the mouse subcutaneous abscess model (J. Liese and R. P. Novick, unpublished data), as well as in biofilm formation (7, 29), so these strain backgrounds would again provide two distinct platforms with which to study differences in virulence and biofilm development associated with the *agr* alleles. Finally, although our focus was on virulence factors regulated primarily through the RNAIII-*rot* axis, an additional area of future experimentation will be to examine the effects of *agr* alleles on the subset of *agr*-dependent genes recently identified to be controlled independently of RNAIII, including the PSM family cytolysins (62).

This study indicates that altered virulon regulation can arise from concerted natural allelic variants in multiple genes, adding to previously established mechanisms of allelic variations in *S. aureus* regulators, including deletion mutants that result in premature protein termination (such as that in *rsbU* [8] and *arlR* [40]), and single-nucleotide polymorphisms resulting in missense substitutions (such as that in *saeS* [2]). Concerted allelic differences in the *agr* loci of other staphylococci and Gram-positive bacteria (20, 78), and in other polymorphic microbial autocrine systems (3, 73), may similarly provide the basis for altered regulatory properties that can affect pathogenicity, in addition to their widely accepted function as determinants of pherotype specificity. As this study demonstrates, the use of congenic strains combined with reporter gene fusions is a sensitive approach to quantitate variations in signaling dynamics among homologous autoinduction systems.

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