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Role of the Accessory Gene Regulator *agr* in Community-Associated Methicillin-Resistant *Staphylococcus aureus* Pathogenesis▽§

Gordon Y. C. Cheung,[1] Rong Wang,[1]† Burhan A. Khan,[1] Daniel E. Sturdevant,[2] and Michael Otto[1]*

Laboratory of Human Bacterial Pathogenesis, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, Bethesda, Maryland 20892,[1] and Research Technologies Branch, Genomics Unit, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, Hamilton, Montana 59840[2]

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The molecular basis underlying the pathogenic success of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is not completely understood, but differential gene expression has been suggested to account at least in part for the high virulence of CA-MRSA strains. Here, we show that the *agr* gene regulatory system has a crucial role in the development of skin infections in the most prevalent CA-MRSA strain USA300. Importantly, our data indicate that this is due to discrepancies between the *agr* regulon of CA-MRSA and those of hospital-associated MRSA and laboratory strains. In particular, *agr* regulation in strain USA300 led to exceptionally strong expression of toxins and exoenzymes, upregulation of fibrinogen-binding proteins, increased capacity to bind fibrinogen, and increased expression of methicillin resistance genes. Our findings demonstrate that *agr* functionality is critical for CA-MRSA disease and indicate that an adaptation of the *agr* regulon contributed to the evolution of highly pathogenic CA-MRSA.

Pandemic community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains represent a serious problem for the public health system (25). Previously, MRSA infections were reported exclusively in the hospital setting and in immune-compromised or otherwise predisposed patients. In contrast, the recently emerging CA-MRSA strains combine antibiotic resistance with high virulence and transmissibility, enabling them to spread and cause severe infections in otherwise healthy people (13, 33). The most serious CA-MRSA pandemic is seen in the United States, where almost all cases are caused by pulsed-field type USA300 (30), a group of closely related CA-MRSA strains (24) that are now also spreading in other parts of the world (37, 39, 48).

The virulence potential of *S. aureus* is determined mainly by its ability to evade human innate host defenses, among which the interaction with phagocytes, such as neutrophils, plays a preeminent role (12). *S. aureus* has developed many mechanisms to subvert destruction by human neutrophils, such as a protective capsule or enzymes that eliminate substances produced by neutrophils to kill the bacteria (18). In addition, *S. aureus* may secrete toxins that lyse human neutrophils and other immune cells, such as alpha-toxin, phenol-soluble modulins (PSMs), and members of the leukocidin family, which include Panton-Valentine leukocidin (PVL) (12, 18). Furthermore, surface proteins that, among other tasks, facilitate adhesion to host tissue are believed to contribute significantly to the establishment of *S. aureus* infections (11).

Virtually all *S. aureus* toxins are under the control of the pivotal virulence regulator *agr* (31). This system triggers pronounced changes in gene expression at a certain level of cell density by a process called quorum sensing. In addition to toxins, *agr* is known to upregulate a wide variety of virulence determinants, such as exoenzymes (proteases, lipases, nucleases), and downregulate expression of surface binding proteins. This adaptation is believed to ascertain production of specific sets of virulence determinants of an infection, when they are most needed: binding proteins at the beginning, when cell density is low and adhesion to host tissue is crucial, and toxins and degradative exoenzymes when the infection is established, nutrients need to be acquired from host tissues, and the concomitant activation of the host's immune system requires production of immune evasion factors. The timely activation of *agr in vivo* and its importance for virulence of *S. aureus* have been demonstrated (8, 22, 49), even though these studies often used only laboratory strains. The role of *agr* in clinical strains of *S. aureus* is less well understood. Particularly, no reports about the impact of *agr* on the virulence of CA-MRSA have been published.

Previous work indicated a crucial role of differential gene expression in the evolution of CA-MRSA virulence and suggested that *agr* control has a major function in establishing the exceptional virulence potential of the most predominant CA-MRSA strain USA300 (27, 29, 33, 45). Thus, we investigated the role of *agr* in the CA-MRSA strain USA300 in detail, using an animal skin infection model and genome-wide analysis of *agr*-dependent gene expression in comparison with hospital-associated MRSA (HA-MRSA) and laboratory strains. We demonstrate a strong impact of *agr* on CA-MRSA (USA300) virulence in experimental skin infection and provide evidence indicating that an adaptation of *agr*-dependent gene regulation

* Corresponding author. Mailing address: Laboratory of Human Bacterial Pathogenesis, NIAID, NIH, Bldg. 33, 1W10, 9000 Rockville Pike, Bethesda, MD 20892. Phone: (301) 443-5209. Fax: (301) 480-3633. E-mail: motto@niaid.nih.gov.

† Present address: Meat Safety and Quality Research Unit, U.S. Meat Animal Research Center, P.O. Box 166, Spur 18D, Clay Center, NE 68933.

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TABLE 1. Primers and probes used for RT-PCR

Gene	Forward primer	Reverse primer	Probe
clfB	TTCCAATGCGCAAGGAACTAG	CAGCATTTACTACAGGTTCAGC AACT	AGACTACGTACAGCTCTCGTTCTAAC ACTT
gyrB	CAAATGATCACAGCATTTGGT ACAG	CGGCATCAGTCATAATGACGAT	AATCGGTGGCGACTTTGATCTAGCG AAAG
hla	AAAAAACTGCTAGTTATTAGA ACGAAAGG	GGCCAGGCTAAACCACTTTTG	CCTTCTTCGCTATAAACTCTATATTGACC AGCAAT
rnaIII	GTGATGGAAAATAGTTGATG AGTTGTTT	GAATTTGTTCACTGTGTCGATA ATCC	TGCACAAGATATCATTTCAACAATCAGTG ACTTAGTAAAA
sdrD	TCAGATGAGCAAGCTTCA CCAA	TTGGTTGAGCATTTACCACTGATT	ATTCTCTTGCAAATCAGGTTGTAACGCTT CTTG
rot	ATTTTGCAATTAGAAACACTT TTGG	TCTTCTCTAGACATTTTGTATTCG CTTT	TGACATTAACTCAATTTTCAGCGAGATTG
sarH1	CCACCATAAATACCCTCAAAC TGTT	TCATCTTCAGTTGAGCGTTCTTTT	AGCTCTCAATAATTTAAAAAAGCAAG GCTA
sdrC	CAACTGCAGATCAGCCTAAA GTGA	TGGTGATTGCATGTTACTACTAGT TTCTT	TGAGTGATAGTGCAACAGTT
clfA	AGGTTCTGGTGACGGTATCGA	TCAATTTCACCAGGCTCATCAG	AAACCAGTTGTTCCTGAAC
efb	TTTAACGATGGTACATTCGAA TATGG	ATCAGTTTTCGCTGCTGGTTTAT	CACGTCCACAATTT
mecA	TTCCACATTGTTTCGGTCTAA AATT	AATGCAGAAAGACCAAAGCA TACA	CCACGTTCTGATTTTAAA
arcA1	TGCGATCGTATGTCACCACAA	CAATGGAATGATGGCTCAAACA	CCTGGTCGAATACATAAT
arcA2	TCAGCTGCTAACTTCTCAAGG TAAAG	CATTTTGCGCAGGTGCTAAG	ACTTCAACACCCTCTTC
spa	CAGCAAACCATGCAGATGCTA	GCTAATGATAATCCACCAAATAC AGTTG	CATTACCAGAAACTGGTGAAGAAAATCC ATTCATTG

contributed to the evolution of virulence in the CA-MRSA strain USA300.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. aureus* strains used in this study were LAC (CA-MRSA, pulsed-field type USA300) (6), MW2 (CA-MRSA, pulsed-field type USA400) (1), Sanger 252 (HA-MRSA, pulsed-field type USA200) (21), and RN6390 (laboratory strain, derived from strain 8325) (32). RN6390 and USA300 both belong to sequence type 8. Deletion mutants in the *agr* regulatory system were produced by transduction of the transposon insertion from strain RN6911 in this study (strain 252) or previously (4). The *agr* system is entirely deleted in these strains, except for a 3′ part of RNAIII, which is not transcribed owing to the absence of the corresponding promoter. All mutants were verified by analytical PCR, DNA sequencing of the flanking regions, and real-time PCR (RT-PCR) for absence of the RNAIII transcript. Main cultures for microarray analysis and quantitative RT-PCR (qRT-PCR) were inoculated from precultures grown overnight to an optical density at 600 nm (OD_{600}) of 0.1.

RNA isolation, transcriptional profiling, and qRT-PCR. RNA isolation from cultures grown to the indicated time points, removal of remaining DNA, cleanup, cDNA synthesis, and labeling were performed as previously described (28). Biotinylated *S. aureus* cDNA was hybridized to custom Affymetrix GeneChips (RMLChip 7) with 100% coverage of chromosomal genes from USA300 and scanned according to standard GeneChip protocols (Affymetrix). Each experiment was replicated three times. Affymetrix GeneChip Operating Software (GCOS; version 1.4) was used to perform the preliminary analysis of the custom GeneChips at the probe set level. Subsequent data analysis was performed as described previously (28). The complete set of microarray data was deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO Series accession number GSE18793. Quantitative RT-PCR was performed as described previously (26), with at least three independent samples. All oligonucleotides were synthesized by Sigma (Table 1).

Fibrinogen adhesion assay. To measure adhesion to fibrinogen, microtiter plates were coated with 100 μl/well of a 10-μg/ml fibrinogen suspension at 4°C overnight. Afterward, an equal volume of a 5-mg/ml bovine serum albumin (BSA) solution was added and the plates were incubated at 37°C for 2 h. Plates were washed four times with phosphate-buffered saline (PBS), after which 100 μl of bacterial solutions normalized to an OD_{600} of 1 were added. Plates were incubated at 37°C for 1 h. Adhered bacteria were counted using microscopy.

Mouse subcutaneous infection model. The subcutaneous abscess model was performed as described previously (45). Briefly, Crl:SKH1-hrBR hairless mice were between 4 and 6 weeks of age at the time of use. Mice were inoculated with *S. aureus* from mid-exponential growth phase (3 h) with the indicated number of CFU in 50 μl as described previously (44). Animals were examined for skin lesions at 24-h intervals for a total of 14 days. Skin lesion dimensions were measured daily with a caliper. Length (L) and width (W) values were applied to calculate the area of lesions using the formula of $L \times W$. All animals were euthanized after completion of the entire procedure. Animal studies were approved by the Animal Care and Use Committee (IUCAC number ASP LHBP 1E), National Institute of Allergy and Infectious Diseases.

RESULTS

Impact of *agr* on the outcome of skin infection caused by CA-MRSA. To investigate the role of *agr* during infection, we performed a mouse subcutaneous infection model, which mimics skin and soft tissue infections as the most common manifestation of disease caused by CA-MRSA (30) (Fig. 1). Subcutaneous injection of the same numbers of bacteria ($\sim 3 \times 10^7$) resulted in strong abscess formation using strain LAC (USA300), while abscesses were significantly smaller with the LAC *agr*, 252, and 252 *agr* strains (Fig. 1A). Furthermore, animals infected with the LAC strain developed necrosis of the epidermis and dermis with epidermal ulceration (Fig. 1B and C). In contrast, the LAC *agr*, 252, and 252 *agr* strains caused chronic, subcutaneous abscesses lacking the dermal and epidermal necrosis noted with the LAC strain. Mice infected with the laboratory strain RN6390 (at 10^6 CFU) developed abscesses that were dermonecrotic and larger than those in mice infected with RN6911 (*agr*), which never showed necrosis. Finally, animals injected with PBS control or heat-killed cells of any of the strains used did not

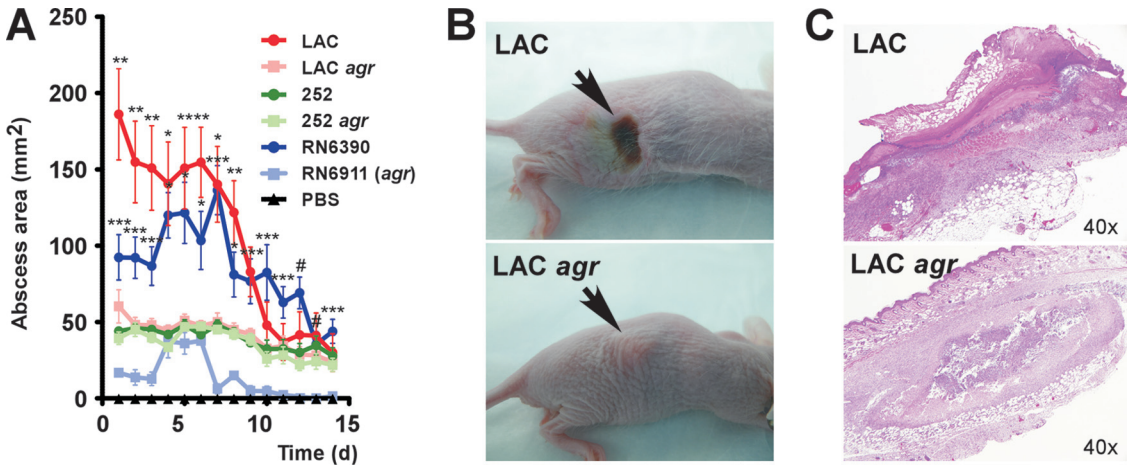

FIG. 1. Effect of *agr* on virulence in a mouse subcutaneous infection model. (A) Abscess sizes. Bacteria of the indicated strains were injected subcutaneously in hairless mice at ~3×10^7 CFU (except RN6390 and RN6911, 1×10^6 CFU), and abscess dimensions were measured every day. Statistical significance of differences between abscess sizes on each day for each of the three wild-type/*agr* mutant comparisons was determined using unpaired *t* tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, all values achieved with the *agr* mutant strain were 0. (B) Development of dermonecrotic lesions. Mice infected with the LAC and RN6390 strains, but not the other strains, commonly developed open dermonecrotic lesions as shown in the upper panel. The lower panel shows a characteristic closed abscess formed by the LAC *agr* strain. (C) Histopathological evaluation of abscesses formed by the LAC and LAC *agr* strains. Histopathology of abscesses formed by RN6390 was similar to that of abscesses formed by strain LAC, and histopathologies of abscesses formed by the 252, 252 *agr*, and RN6911 strains were similar to those of abscesses formed by the LAC *agr* strain.

develop abscesses, supporting the idea that bacterial production of toxins rather than the host's response to proinflammatory components of the staphylococcal surface plays a major role in the development of CA-MRSA-induced skin disease. These results demonstrate that the *agr* system has a crucial impact on the development of skin infections by the CA-MRSA strain USA300.

***agr* activity.** To investigate the mechanistic basis of the impact that the *agr* system has on the virulence of strain USA300, we first determined *agr* activities during *in vitro* growth (32, 35). While maximal *agr* activity in strain 252 was reached 2 h later than that in strains USA300 and RN6390, most likely owing to slower growth and delayed onset of the postexponential growth phase in that strain, we found that maximal activities of *agr* in the LAC and 252 strains were not significantly different ($P = 0.55$, Student's *t* test) (Fig. 2). This indicates that the significantly lower virulence potential of strain 252 and the observed differential impact of *agr* on virulence in these two strains are not due primarily to differences in *agr* activities.

***agr*-dependent gene regulation in CA-MRSA: overview, toxins, and exoenzymes.** It has been reported that the *agr* regulon in clinical strains may differ from the scheme of *agr* regulation that had been established using laboratory strains (2, 35). This prompted us to evaluate the hypothesis that the *agr* regulons rather than *agr* activities may account for the differential impact *agr* has on the virulence potential in CA- versus HA-MRSA. To that end, we analyzed the *agr* regulons of the LAC, 252, and RN6390 strains by genome-wide transcriptional profiling.

Analysis of the total up- and downregulated genes did not reveal pronounced differences: strain 252 showed 360 differentially regulated genes, strain LAC showed 262, and strain RN6390 showed 228. Forty-three genes were differentially regulated in all three strains. Genes coding for proteases, lipases, phenol-soluble modulins (PSMs), and other toxins were strongly upregulated by *agr* in all three strains (Table 2; see also Table S1 in the supplemental material). The most strongly regulated toxin genes in strain LAC were those coding for the Panton-Valentine leukocidin (PVL) and PSMs. While the microarray contained only samples for the *psm*β genes, owing to the small size of the other *psm* genes, qRT-PCR demonstrated exceptionally strong *agr* control and exceptionally high expression in strain LAC also for the *psm*α genes (Fig. 3A). Furthermore, the strong impact of *agr* on PSM expression was verified on the protein level for all PSMs (Fig. 3B). In accordance with previous results (45), expression of PSMs was overall lower in the 252 strain compared to strain LAC. Production of the PSMα peptides, the major contributors among PSMs to CA-MRSA skin infection and bacteremia (45), was considerably higher in strain LAC than in strains 252 and RN6390, in both absolute and relative terms. Moreover, upregulation by *agr* of secreted degradative enzymes, such as lipases and proteases, was much stronger in strain LAC than in strain 252, while further virulence genes, such as *lrgA* and *lrgB*, involved in bacterial programmed cell death (36) and staphylokinase (*sak*) (3) were downregulated by *agr* in strain 252 but not in strain LAC. While the *hla* (alpha-toxin) gene is not functional in strain 252 owing to a nonsense mutation (5), it is still interesting to note that *hla* was upregulated by *agr* in strain LAC while downregulated in strain 252. Data achieved using qRT-PCR and Western blot analysis confirmed a strong impact of *agr* on *hla* expression in strain LAC (Fig. 4). Remarkably, the relative abundances of *hla* transcript and alpha-toxin in the six investigated strains were similar to the differences in virulence that we observed in the skin infection model, in keeping with the notion that alpha-toxin is a key virulence determinant in CA-MRSA

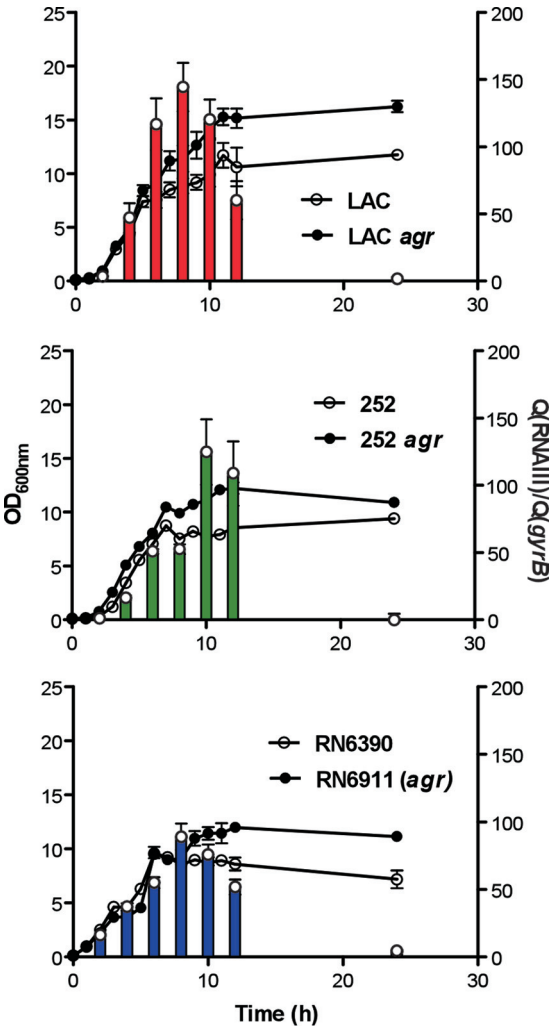

FIG. 2. Growth-dependent expression of *agr*. Expression of *agr* was determined using qRT-PCR of RNAIII during the growth of *S. aureus* strains and their isogenic *agr* mutants. Lines represent OD_{600}, plotted on the left *y* axis. Bars represent the relative expression of RNAIII compared to that of the housekeeping gene *gyrB* (control), plotted on the right *y* axis. Expression of RNAIII in *agr*-negative strains was not detectable in any strain at any time point.

skin infections (23) and serving as an explanation for the high virulence observed for strain RN6390.

Methicillin resistance. So far, no report describing an impact of *agr* on methicillin resistance exists, except for our previous observation of *agr*-dependent expression of *mecA* in the CA-MRSA strain MW2 (USA400) (35). Here, we found that genes involved in the resistance to methicillin (*mecA*, *mecR1*) were strongly upregulated by *agr* in the CA-MRSA strain but much less in the HA-MRSA strain, which was confirmed by qRT-PCR of the *mecA* gene (Fig. 4). In accordance with the idea that *agr* impacts methicillin resistance in strain LAC, oxacillin had a stronger growth impact on the LAC *agr* than the LAC wild-type strain at low concentrations, while no impact of *agr* on methicillin resistance was detectable for strain 252 (Fig. 5A). These results show that *agr* has a gene regulatory effect on the expression of methicillin resistance genes and the methicillin resistance phenotype in CA-MRSA.

Surface binding proteins and fibrinogen binding capacity. According to microarray and qRT-PCR analyses, expression of surface binding protein genes in strain LAC differed significantly from that of the other strains and only partially followed the general dogma of *agr* regulation, which predicts a negative effect of *agr* on the expression of those genes. First, transcription of *sdrC*, *sdrE*, *fnbA*, *fnbB*, and *empbp* was either only moderately downregulated by *agr* in strain LAC or not affected (Table 2). Only with the protein A gene *spa* did we observe the predicted strong negative *agr* effect (Fig. 4). Notably, strong expression and positive rather than negative regulation by *agr* of the fibrinogen binding protein genes *clfA* and *efb* in strain LAC were in clear contrast to the low expression of surface binding protein genes in the other wild-type strains and the notion about commonly negative regulation of this class of genes by *agr*. Fibrinogen binding assays confirmed that the observed differences in *clfA* and *efb* expression translated to a high capacity of strain LAC to bind fibrinogen and a strong effect of *agr* on that capacity, which was much lower or absent in the other strains (Fig. 5B). Thus, expression and *agr*-dependent regulation of surface protein genes in strain LAC are profoundly different from those in HA-MRSA and laboratory strains, allowing for a simultaneous expression of surface binding proteins, in particular those involved in binding of fibrinogen (ClfA, Efb), and universally *agr*-upregulated virulence determinants, such as toxins and exoenzymes.

Metabolism and arginine deiminase. The *arc* operon responsible for arginine deiminase activity has been discussed as a potential contributor to pH homeostasis and immune evasion, impacting colonization and virulence properties (17, 41). In addition to the core genome-encoded *arc* operon (*arc2*), the *arc* operon present on the arginine catabolic mobile element (ACME), *arc1*, which among CA-MRSA is uniquely found in USA300 clones, has drawn particular interest, as it may contribute to the exceptional virulence and transmissibility features of these predominant CA-MRSA strains (14, 15, 17). The core genome and ACME-encoded *arc* operons were downregulated by *agr* in USA300, similar to a single, third *arc* gene (*arcC3*), but expression of the *arc* operons was low overall (Table 2, Fig. 4). Interestingly, the sum of *arcA1* and *arcA2* transcript levels in strain LAC was not as high as the level of *arcA2* transcript in strain 252, which is inconsistent with the idea that arginine deiminase activity is responsible for the higher pathogenic potential of LAC than 252. Nevertheless, ACME-encoded *arc* may be important in overcoming to some extent the very limited core genome-encoded *arc* activity in strain LAC.

Additionally, we observed several pronounced differences between the strains regarding *agr*-dependent expression of further genes involved in metabolism (Table 2). While the present evaluation is not meant to focus on metabolism, we noted, for example, a strong upregulation of argininosuccinate lyase and synthase only in strain RN6390 but not in the other strains, while enzymes involved in riboflavin synthesis were upregulated only in strain 252. Formate acetyltransferase *pflB* and pyruvate formate-lyase-activating *pflA* genes were *agr* regulated only in strain 252.

TABLE 2. Selected *agr*-regulated genes in strains LAC, 252, and RN6390

Gene type and no.	Gene	Function	Fold change in gene expression (wild-type/*agr* strain)[a]		
			LAC	252	RN6390
Virulence					
SAUSA300_0113	*spa*	Immunoglobulin G binding protein A	0.12	0.04	0.03
SAUSA300_0256	*lrgA*	Murein hydrolase exporter	NC	0.35	
SAUSA300_0257	*lrgB*	Murein hydrolase export regulator		0.09	
SAUSA300_0320	*geh*	Lipase (EC 3.1.1.3)	6.31	2.49	3.13
SAUSA300_0424		Low-affinity zinc transport protein	7.33		
SAUSA300_0546	*sdrC*	Neurexin binding protein			0.46
SAUSA300_0547	*sdrD*	Surface binding protein	0.17	NP	0.06
SAUSA300_0548	*sdrE*	Surface binding protein			
SAUSA300_0630		Multidrug resistance ABC transporter	7.51	10.26	5.70
SAUSA300_0772	*clfA*	Clumping factor A (fibrinogen binding protein)	2.08	2.57	
SAUSA300_0774	*empbp*	Extracellular matrix binding protein/fibrinogen binding protein	0.36		
SAUSA300_0776	*nuc*	Thermonuclease (EC 3.1.31.1)	0.18	0.27	
SAUSA300_0835	*dltA*	D-Alanine-activating enzyme (EC 6.3.2.-)	0.33	0.46	
SAUSA300_0836	*dltB*	Protein DltB	0.33	0.33	
SAUSA300_0837	*dltC*	D-Alanyl carrier protein	0.43	0.41	
SAUSA300_0838	*dltD*	Protein DltD	0.27	0.39	0.49
SAUSA300_0949	*sspC*	Hypothetical protein	6.79		27.33
SAUSA300_0950	*sspB*	Staphopain (EC 3.4.22.-)	9.74		11.33
SAUSA300_0951	*sspA*	Glutamyl endopeptidase (EC 3.4.21.19)	11.30		9.59
SAUSA300_1055	*efb*	Fibrinogen binding protein	3.00		
SAUSA300_1058	*hla*	Alpha-hemolysin	2.17	0.25	23.68
SAUSA300_1067	*psmβ1*	Phenol-soluble modulin beta 1	173.35	61.61	41.55
SAUSA300_1068	*psmβ2*	Phenol-soluble modulin beta 2	63.24	NP	26.07
SAUSA300_1381	*lukF-PV*	Leukocidin F subunit	7.14	NP	NP
SAUSA300_1382	*lukS-PV*	Leukocidin S subunit	6.52	NP	NP
SAUSA300_1753	*splF*	Serine protease (EC 3.4.21.-)	14.96	3.32	89.05
SAUSA300_1754	*splE*	Serine protease (EC 3.4.21.-)	11.11		33.61
SAUSA300_1755	*splD*	Serine protease (EC 3.4.21.-)	10.88		44.83
SAUSA300_1756	*splC*	Serine protease (EC 3.4.21.-)	9.91		46.21
SAUSA300_1757	*splB*	Serine protease (EC 3.4.21.-)	10.71		64.80
SAUSA300_1758	*splA*	Serine protease (EC 3.4.21.-)	7.99		27.45
SAUSA300_1759		Hypothetical protein	69.53	NC	4.69
SAUSA300_1918	*hlb*	Sphingomyelin phosphodiesterase (EC 3.1.4.12), truncated beta-toxin	6.39		8.85
SAUSA300_1922	*sak*	Staphylokinase		0.49	
SAUSA300_2440	*fnbB*	Fibronectin binding protein	0.24	NP	
SAUSA300_2441	*fnbA*	Fibronectin binding protein			2.87
SAUSA300_2603	*lip*	Lipase (EC 3.1.1.3)	12.54	7.53	13.29
Resistance					
SAUSA300_0032	*mecA*	MecA protein	4.70		NC
SAUSA300_0033	*mecR1*	Methicillin resistance protein	3.02		NC
SAUSA300_0928	*comK*	Competence transcription factor	10.50	2.44	7.25
Metabolism					
SAUSA300_0061	*arcC1*	Carbamate kinase (EC 2.7.2.2) (ACME)		NP	NC
SAUSA300_0062	*arcB1*	Ornithine carbamoyltransferase (EC 2.1.3.3) (ACME)	0.46	NP	NC
SAUSA300_0063		Transcription regulator, *crp* family (ACME)	0.45	NP	NC
SAUSA300_0064	*arcD1*	Arginine/ornithine antiporter (ACME)	0.40	NP	NC
SAUSA300_0065	*arcA1*	Arginine deiminase (EC 3.5.3.6) (ACME)	0.41	NP	NC
SAUSA300_0220	*pflB*	Formate acetyltransferase (EC 2.3.1.54)		0.10	
SAUSA300_0221	*pflA*	Pyruvate formate-lyase-activating enzyme (EC 1.97.1.4)		0.19	
SAUSA300_0311		Ribokinase (EC 2.7.1.15)	0.05	0.06	0.16
SAUSA300_0312		Sugar kinase	0.05	0.04	0.26
SAUSA300_0313		Nucleoside permease *nupC*	0.05	0.05	0.19
SAUSA300_0863	*argH*	Argininosuccinate lyase (EC 4.3.2.1)			58.19
SAUSA300_0864	*argG*	Argininosuccinate synthase (EC 6.3.4.5)			54.46
SAUSA300_1062	*argF*	Ornithine carbamoyltransferase (EC 2.1.3.3)	0.07	0.12	0.31
SAUSA300_1063	*arcC3*	Carbamate kinase (EC 2.7.2.2)	0.08	0.19	0.30
SAUSA300_1712	*ribH*	6,7-Dimethyl-8-ribityllumazine synthase (EC 2.5.1.9)		31.50	
SAUSA300_1713	*ribBA*	GTP cyclohydrolase II (EC 3.5.4.25)/3,4-dihydroxy-2-butanone-4-phosphate synthase (EC 4.1.2.-)		15.58	
SAUSA300_1714	*ribE*	Riboflavin synthase alpha chain (EC 2.5.1.9)		42.18	

Continued on following page

TABLE 2—*Continued*

Gene type and no.	Gene	Function	Fold change in gene expression (wild-type/*agr* strain)[a]		
			LAC	252	RN6390
SAUSA300_1715	*ribD*	Diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26)/5-amino-6-(5-phosphoribosylamino)uracil reductase (EC 1.1.1.193)		24.48	
SAUSA300_2567	*arcC2*	Carbamate kinase (EC 2.7.2.2)		0.15	0.08
SAUSA300_2568	*arcD2*	Arginine/ornithine antiporter		0.15	0.21
SAUSA300_2569	*arcB2*	Ornithine carbamoyltransferase (EC 2.1.3.3)		0.18	0.11
SAUSA300_2570	*arcA2*	Arginine deiminase (EC 3.5.3.6)		0.22	0.05
Regulation					
SAUSA300_0605	*sarA*	Staphylococcal accessory regulator			2.77
SAUSA300_0690	*saeS*	Sensory transduction protein kinase (EC 2.7.3.-)	2.61		
SAUSA300_0691	*saeR*	Two-component response regulator	2.17		
SAUSA300_1708	*rot*	Staphylococcal accessory regulator		0.64	3.18
Transport					
SAUSA300_2453		ABC transporter ATP binding protein	0.05	0.09	0.20
SAUSA300_2454		ABC transporter ATP binding protein	0.06	0.06	0.15

[a] NC, empty fields, no significant change; NP, gene not present in that strain.

Virulence regulators. Expression of several regulatory systems that are reportedly involved in virulence gene regulation was different among the tested strains (Table 2). The *sarA* regulator (9) was affected in the laboratory but not the clinical strains, while upregulation of the *saeRS* regulator (20) occurred only in strain LAC. Interestingly, expression of the *sarA* homolog *rot*, an important mediator of the *agr* regulatory effect (19, 38), differed substantially among the strains. It was not significantly affected in strain LAC, moderately downregulated in strain 252, and upregulated in strain RN6390. While *agr*-dependent control of Rot function occurs predominantly on the posttranscriptional level (19), upregulation of *rot* transcript levels in an *agr*-dependent fashion has been observed previously (19), in accordance with our results. Opposite effects of *agr* on *rot* transcript levels between laboratory and clinical strains were confirmed by qRT-PCR (Fig. 4), an observation with potentially great importance for the difference in virulence gene regulation between those strains.

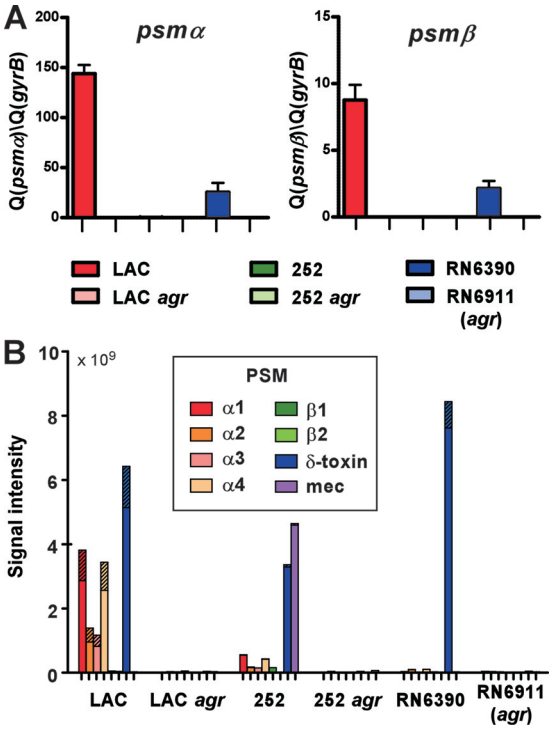

FIG. 3. Effect of *agr* on PSM expression in various strain backgrounds. (A) Expression of the *psm*α and *psm*β operons was determined using qRT-PCR as previously described (35). (B) Concentrations of all *S. aureus* PSMs (see legend) in culture filtrates were measured as described previously (35). Samples for measurements were taken from 8-h (strains LAC and RN6390) or 10-h (strain 252) cultures. Striped parts of bars represent the *N*-deformylated fraction of a particular PSM. Note that strain 252 contains the *psm-mec* gene in contrast to strains LAC and RN6390.

DISCUSSION

One of the most challenging tasks in current research on staphylococcal diseases is to elucidate the basis of the exceptional virulence potential of CA-MRSA strains, particularly the most epidemiologically successful clone USA300 (15). Acquisition of mobile genetic elements (MGEs), such as ΦSLT, which carries the genes encoding PVL, and ACME, which comprises two operons involved in arginine metabolism and oligopeptide transport, has been deemed a crucial step during the evolution of the virulence of USA300 (15, 16, 42). While ACME is found only in USA300, the *lukSF* genes encoding PVL are also found in many other CA-MRSA strains, and a correlation between CA-MRSA infection and the presence of the *lukSF* genes has been noted (42). However, more recent research has questioned the importance of PVL as a major contributor to CA-MRSA virulence (43, 44, 46, 47). Furthermore, while ACME may have other roles that promote the epidemiological success of USA300 (15), such as in facilitating colonization, the contribution of ACME to virulence is minor (17). Alternatively, or additionally, genetic rearrangements leading to increased expression of core genome-encoded gene products may have contributed to the evolution of CA-MRSA

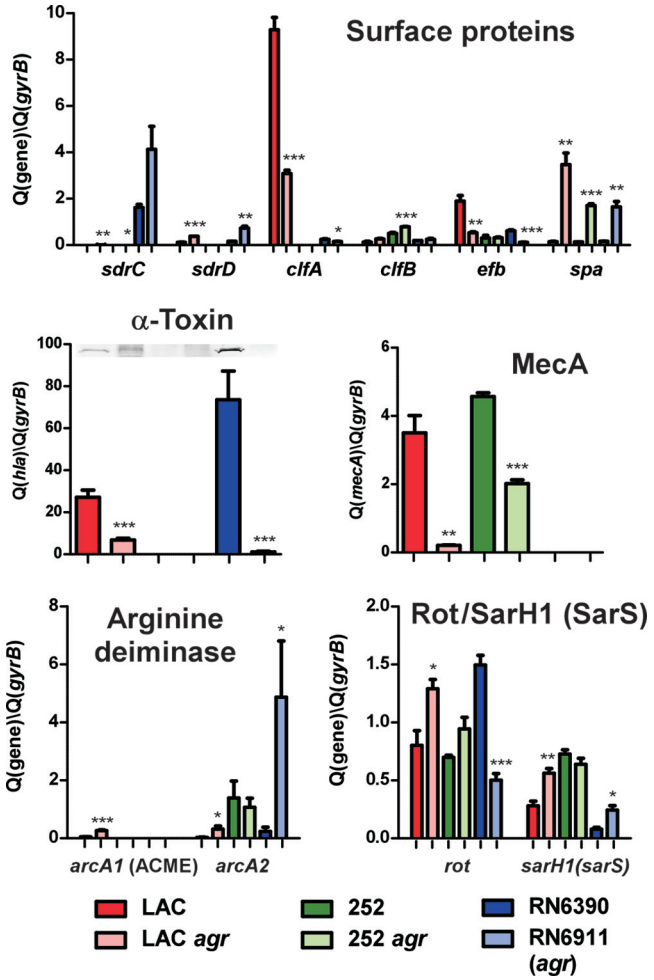

FIG. 4. Effect of *agr* on the expression of selected surface protein and regulatory genes in various strain backgrounds. The graphs show expression of selected genes as determined by qRT-PCR at maximal expression of *agr* in the LAC, LAC *agr*, RN6390, and RN6911 strains for 8 h and the 252 and 252 *agr* strains for 10 h. Data shown on the *y* axis represent expression relative to that of the housekeeping gene *gyrB* (control). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*t* tests comparing *agr*-negative to wild-type strain samples). Expression levels of alpha-toxin were confirmed on the protein level by Western blots with specific alpha-toxin antiserum and are shown above the qRT-PCR data.

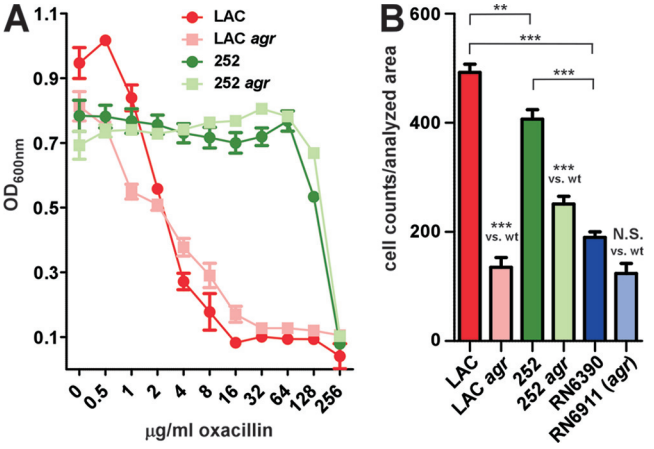

FIG. 5. Effect of *agr* on methicillin resistance and fibrinogen binding phenotypes. (A) MIC assays with oxacillin. Strains were inoculated from precultures and grown with the addition of oxacillin at different concentrations in microtiter plates for 24 h. (B) Fibrinogen binding capacity. Bacteria were grown for 16 h and assayed for fibrinogen binding capacity on fibrinogen-coated microtiter plates after an incubation period of 2 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

virulence (15). Indeed, recent results from our laboratory have emphasized the importance of differential gene expression for the evolution of virulence within the clonal complex 8 subtree that includes USA300 (27). In the present study, we observed a dramatic impact of *agr* on the development of experimental skin disease caused by strain USA300, confirming our hypothesis on the presumed key role of *agr* in CA-MRSA skin infections (27). Of note, these results underline the potential of drugs interfering with the function of *agr* (22, 34). Furthermore, we demonstrated that the *agr* regulon of strain USA300 shows important discrepancies compared to those of HA-MRSA and laboratory strains, indicating a possible role of these adaptations in the contribution of *agr* to CA-MRSA pathogenesis.

Transcriptional profiling analysis and qRT-PCR indicated strong *agr* regulation of important toxins in the CA-MRSA USA300 strain, including the most frequently proposed candidates for proteins with a major contribution to CA-MRSA virulence: alpha-toxin, PVL, and PSMs. Furthermore, we found differences in the *agr* regulons that may explain strain differences in virulence even at the observed comparable overall activity of *agr* between standard CA- and HA-MRSA strains. These included much more pronounced upregulation of toxins and lytic enzymes in USA300 compared to that of the HA-MRSA 252 strain (Table 2 and Fig. 3 and 4). Moreover, in contrast to the established scheme of *agr*-dependent gene regulation, high activity of *agr* did not cause, or only resulted in moderate, downregulation of surface protein gene expression, with the exception of the protein A gene *spa*. Of particular interest, the *clfA* and *efb* fibrinogen binding protein genes were highly expressed and upregulated by *agr* in strain USA300, as was fibrinogen binding capacity in that strain. Positive regulation by *agr* is in accordance with the described roles of fibrinogen-binding proteins, such as ClfA, in the early dissemination phase of *S. aureus* infection (7) and high activity of *agr* in the early infection phase (49). Comparatively high expression of those proteins in CA-MRSA may thus contribute to the exceptional capacity to infect and spread systemically, a hypothesis that needs to be further evaluated.

We previously noted that the *mecA* gene responsible for methicillin resistance in MRSA is under the control of *agr* in the CA-MRSA strain MW2 (35). In the present study, we observed similar control in the CA-MRSA LAC strain, with the effect of *agr* on *mecA* expression and methicillin resistance being more pronounced than in the HA-MRSA 252 strain. The regulatory interdependence of the *mec* and *agr* systems may contribute to balancing these two important energy-consuming pathogenesis mechanisms.

Taken together, our findings suggest that evolution of virulence of USA300 proceeded via adaptation of the *agr* regulatory network, resulting in the potential to maintain production

of commonly *agr*-downregulated factors, such as surface proteins, while simultaneously ensuring secretion of aggressive toxins and promoting methicillin resistance, thereby optimizing gene expression for immune evasion, tissue adhesion, and antibiotic resistance. At present, the molecular nature of these regulatory adaptations is not clear. Comparing strains USA300 and 252, we did not detect differences that could clearly be made responsible for the *agr* regulon changes at the level of the regulator molecules, notably including both the RNAIII- and AgrA-regulated parts of the *agr* regulon (35). Namely, in strain USA300 compared to strain 252, there was no difference in the AgrA amino acid sequence: only one conservative amino acid exchange (I20M), which was not unique among *S. aureus* strains for either strain, in the Rot sequence, and only a 1-base-pair change within RNAIII (deletion of one T in a low-complexity 8-T sequence). We previously speculated that SarH1 (SarS) (40), the only SarA paralog (10) found to be *agr* regulated in strain MW2, might substitute for the pivotal role of Rot in *agr*-dependent target gene regulation (35). However, *sarH1* (*sarS*) expression data did not confirm the hypothesis that *agr* regulon diversity is due to differential expression of SarH1/SarS (Fig. 4). Together with the microarray data, these observations suggest that adaptations may have occurred at the target gene level. In the future, a detailed investigation of regulatory networks and regulated targets in USA300 will be needed to further elucidate the interesting phenomenon of *agr* regulon diversity and its impact on the evolution of virulence in CA-MRSA.

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