The role of ArlRS in autolysis in methicillin-sensitive (MSSA) and methicillin-resistant \textit{Staphylococcus aureus} strains (MRSA)

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Abstract

Autolysis plays an essential role in bacterial cell-division and lysis with β-lactam antibiotics. Accordingly, the expression of autolysins is tightly regulated by several endogenous regulators including ArlRS, a two component regulatory system that has been shown to negatively regulate autolysis in methicillin-sensitive *S. aureus* strains (MSSA). In this study, we found that inactivation of *arlrS* does not play a role in autolysis of methicillin resistant strains (MRSA) such as CA-MRSA strains USA300 and MW2 or the HA-MRSA strain COL. This contrasts with MSSA strains including Newman, SH1000, RN6390 and 8325-4 where autolysis is affected by ArlRS. We further demonstrated that the striking difference in the role of *arlrS* between MSSA and MRSA strains is not due to the methicillin-resistance determinant *mecA*. Among known autolysins and their regulators, we found that *arlrS* represses *lytN* while no effect was seen on *atl*, *lytM* and *lytH* expression in both CA- and HA-MRSA strains. Transcriptional fusion assays showed that the *agr* transcripts, RNAII and RNAIII, were significantly more down-regulated in the *arlrS* mutant of MW2 than the MSSA strain Newman. Importantly, provision of *agr* RNAIII in trans to the MW2 *arlrS* mutant via a multi-copy plasmid induced autolysis in this MRSA strain. Also, the autolytic phenotype in the *arlrS* mutant of MSSA strain Newman could be rescued by either a mutation in *atl* or *lytM*. Together, these data showed that ArlRS impacts autolysis differently in MSSA and MRSA strains.
Introduction

*Staphylococcus aureus*, classically described as a nosocomial pathogen, has now surfaced as a common cause of community-acquired infections, primarily due to the emergence of strain USA300 and, to a lesser extent, USA400 (5). Recent reports suggest that community-acquired methicillin resistant *S. aureus* (CA-MRSA) infections can occur in the hospital environment (6, 27, 36, 43). The potential replacement of HA- with CA-MRSA strains in nosocomial infections is of concern because CA-MRSA strains appear to be more virulent than HA-MRSA. In addition, treatment options have been hampered due to a reduction in the efficacy of antibiotics.

Autolysis, playing an essential role in cell wall turnover, can be triggered by antibiotics or adverse physiological conditions (29, 45). *S. aureus* is known to carry several known or putative autolysins (or peptidoglycan hydrolases) including the major autolysin *atl*, *lytM*, *lytH* and *lytN*, none of which are essential for viability. The major autolysin Atl is a 138-kDa bifunctional protein that undergoes proteolytic processing to generate a 62-kDa N-acetylmuramyl-L-alanine amidase and a 51-kDa endo-β-N-acetylglucosaminidase (26, 35). LytM is a 32-kDa glycyl-glycine endopeptidase that cleaves the pentaglycine linkage between peptidoglycan chain, in a manner similar to lysostaphin (11, 34, 38). LytH (30 kDa), LytN (46 kDa) and LytA (50 kDa) are peptidoglycan hydrolases with N-acetylmuramyl-L-alanine amidase activity (16, 24, 44, 48).

For cell wall homeostasis, autolysin expression is generally tightly regulated. Negative regulators for autolysis include ArlRS, MgrA, LytSR, ClpP, SarA and SarV while *agr* and CidABC are positive regulators (14, 15, 22, 28, 30, 32, 39, 46). In this
study, we focused on the two-component regulatory system ArlRS which was previously shown to be a repressor of autolysis in the methicillin-sensitive *S. aureus* (MSSA) strain 8325-4 (14). In this paper, we decided to examine the role of ArlRS in clinically-relevant MRSA strains. We found that the negative regulation of autolysis by ArlRS occurs in MSSA strains including Newman, 6390, SH1000 and 8325-4 but not in any of the MRSA strains selected for this study including HA-MRSA strain COL and CA-MRSA strains MW2 (USA400) and USA300. This difference in autolysis between MSSA and MRSA strains was not attributable to *mecA*, but can be explained in part by a difference in *agr* expression between these two strain sets. Importantly, provision of *agr in trans* to the arlRS mutant of MW2 via a multi-copy plasmid with an exogenous promoter induced autolysis in this mutant. These studies support the divergent role of ArlRS in inducing autolysis in MRSA and MSSA strains.

**Materials and Methods**

*Bacterial strains and culture conditions.* The *E. coli* strain XL-1 blue was used in cloning experiments. The wild-type and mutant *S. aureus* strains used in this study are listed in Table 1. Luria-Bertani medium (Becton Dickinson) was used for culture of *E. coli* while TSB was used for growth of *S. aureus*. When appropriate, antibiotics (Sigma) were added to the media at the following concentrations: ampicillin at 100 μg/ mL for *E. coli*; chloramphenicol at 10 μg/ mL and erythromycin at 2.5 μg/ mL for *S. aureus*. Chloramphenicol was routinely used to maintain selection for pEPSA5- and pSK236-based plasmids.
Susceptibility testing. Minimum inhibitory concentrations (MIC) were determined in triplicate by microdilution techniques, using an inoculum of $5 \times 10^5$ CFU/mL according to the CLSI guideline (23). For each mutant, three independent clones were tested, with the MIC data reported as median values from at least three independent experiments for each antibiotic. Strains containing pEPSA5-based plasmids were tested with and without xylose induction, but chloramphenicol was not added in these assays to avoid interference with β-lactam resistance evaluation.

DNA techniques. Standard molecular cloning techniques were used as described (40). Restriction enzymes and ligases were from New England Biolabs. I-Proof DNA polymerase (BioRAD) was used to generate all DNA fragments for gene deletions, promoter fusions and ectopic expressions in pEPSA5. The fidelity of all DNA sequences generated by PCR was verified by fluorescently-labeled dideoxynucleotide sequencing (Big Dye™ terminators, PE Applied Biosystems).

Construction of S. aureus mutants. All mutants were generated with in-frame deletion of target genes by allelic replacement, using the temperature-sensitive plasmid pMAD as described (31). This method allows the disruption of genes without the insertion of an antibiotic resistance marker. Briefly, $\sim0.8$ kb PCR products upstream and downstream of targeted sequences were cloned into pMAD, amplified in E. coli, transformed into S. aureus RN4220 and then into the target strain followed by the process of allelic replacement as delineated previously (1). All chromosomal deletions were verified by PCR and DNA sequencing. The resulting deletion strains were devoid of both arlR and
arlS ORFs, with at least three clones each used for characterization. The same pMAD system was also utilized to reinsert the native arlRS ORFs into the MW2 ΔarlRS for complementation. Complementation was also attempted by cloning arlRS in the xylose inducible vector pEPSA5. The sequences of DNA primers used in this study are available from the authors.

Isolation of RNA and Northern Blot Hybridization. Overnight cultures of S. aureus were diluted 1:100 in 40 mL of TSB broth and grown in 200 ml flask with shaking to reach exponential phase (A650nm = 0.7 in Spectronic 20), using 18 mm borosilicate glass tubes. At OD 0.7, total RNA was extracted from 10 mL of culture by using Trizol-glass bead method as described previously (30). After growing for an additional 60 minutes, RNAs were extracted from all samples. Ten micrograms each of total RNA was analyzed by Northern blotting with gel purified DNA probes (∼350 bp each) radiolabeled with [α-32P]-dCTP using the random-primed DNA labeling kit (Roche Diagnostics GmbH) and hybridized under aqueous phase conditions at 65°C. The blots were subsequently washed and bands visualized by autoradiography. Three independent clones were utilized to extract RNA from each strain and tested in Northern blot analysis.

Transcriptional fusion studies of P3 agr promoter linked to the GFP_uvr reporter gene. To confirm the effect of the arlRS deletions on agr transcription, we cloned the agr P3 promoter in pALC1484, a derivative of pSK236 containing the promoterless gfp_uvr to generate transcriptional fusions, as confirmed by restriction analysis and DNA sequencing. Recombinant promoter fusion plasmids were introduced into S. aureus
RN4220, purified and electroporated into wild-type MW2, Newman and their isogenic arlRS mutants. For analysis, aliquots (200 μl) from overnight cultures of three independent clones were transferred to microtiter wells to assay for cell density (OD₆₅₀) and fluorescence in a FL600 fluorescence reader (BioTek Instruments). Promoter activation was plotted as mean fluorescence/OD₆₅₀ ratio, using the average values from triplicate readings from three clones per strain.

Ectopic gene expression in S. aureus. Besides pMAD-mediated complementation of the mutant strains, we also utilized the plasmid pEPSA5 which can be induced for expression with xylose (1%) (10). For pEPSA5 mediated expression, PCR-amplified gene fragments were ligated into pEPSA5, transformed first into E. coli XL-1 blue and then into RN4220. Plasmids from positive clones of S. aureus RN4220 were then introduced into MW2, Newman and their isogenic arlRS mutants as described above. For RT-PCR, extracted RNA was resuspended in DEPC water, treated with Turbo DNase I (Ambion) and then reverse-transcribed with 1 μg of total RNA, using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Regular PCR was then performed on c-DNAs using oligos specific for each gene. For agr over-expression, we utilized pRN6735, (47) which is a derivative of pC194 containing the pI258 bla promoter (P-bla) and two-thirds of the structural gene (blaZ) followed by the 1,566-bp MboI fragment containing the RNAIII determinant lacking its own promoter. The cloned bla promoter is repressed by pI524 in the tested strain, which supplies the bla repressor in-trans. Under these conditions, the bla promoter is inducible by β-lactam compounds such as oxacillin.
X-100 induced autolysis assays in growing cultures. The autolysis assay was performed as described (22). Briefly, overnight cultures were diluted 1:100 to OD$_{650}$ of 0.1 in TSB and grown at 37°C with shaking in the presence of Triton X-100, with OD$_{600}$ recorded hourly for 7–8 h. Each data point represents the mean and standard deviation from three independent experiments.

Zymogram Analysis. Zymogram analysis was conducted to detect alterations in autolysin activity as described with minor alterations (22). Cell fractions containing autolytic enzymes from MW2, Newman and their isogenic ΔarlRS mutants were extracted from the cell pellet of a 10 mL bacterial culture grown to OD$_{650nm}$ = 0.7 using 100 μl of 4% SDS. Equivalent amounts of extracted proteins were separated on the SDS-PAGE gel containing heat-killed RN4220 or *Micrococcus luteus* bacterial cells at 10 mg/ml wet weight. Resolved proteins were allowed to renature overnight in water and incubated with 0.1% methylene blue to visualize clear bands, representing an area of cell lysis with RN4220 or *Micrococcus luteus*. The assay was repeated three times with a representative experiment shown.

Results

Initial characterization of arlRS mutants in autolysis. To assess the contribution of ArlRS to autolysis, we generated in-frame deletion mutants of both the histidine kinase and the response regulator in HA-MRSA strains COL, CA-MRSA strains MW2 and USA300 and MSSA strains Newman, SH1000, 8325-4 and RN6390 (Fig. 1C). We found that MSSA strain Newman and MRSA strain MW2 and their corresponding arlRS
mutants grew comparably in TSB media (Fig. 1A), similar to the growth of arlRS mutants and respective parents in other MSSA and MRSA strains under study (data not shown). In the presence of Triton X-100, however, growth of all arlRS mutants in the MSSA background (8325-4, RN6390, Newman and SH1000) was severely affected while all arlRS mutants of MRSA strains (COL, MW2 and USA300) remained unaffected (Fig. 1B and data not shown). Given the above findings, we have elected to focus most of our ensuing studies on MW2 and Newman, representing MRSA and MSSA strains, respectively.

To exclude chromosomal rearrangement or an ectopic point mutation as the cause for the increased autolysis, the pMAD plasmid was also used to introduce the native arlRS genes into the ΔarlRS mutant of Newman and MW2. These chromosomally complemented strains regained wild type levels of growth in the presence of Triton X-100 (Fig. 2A). Complementation was also obtained when arlRS were cloned into the xylose inducible plasmid pEPSA5 (Fig. 2A), but not when arlR or arlS were singly expressed, possibly due to the high-copy number of the sensor or the kinase gene provided by the plasmid or that ArlR requires ArlS to function properly. We also examined the effect of arlRS mutations on genes upstream and downstream of arlRS in both MW2 and Newman arlRS mutants by Northern blots and did not discern any significant polar effects as a result of the arlRS mutations (data not shown).

Given that autolysis might affect methicillin resistance, we also assayed sensitivity of the arlRS mutants to oxacillin and nafcillin in both strains MW2 and Newman. Deletion of arlRS in both MW2 and Newman did not lead to a significant
reduction in the MIC for oxacillin, nafcillin or cefoxitin which were the β-lactams
selected for this study.

Effect of arlRS on zymographic profiling of autolysins in arlRS mutants. Penicillins and
β-lactams, in general, may enhance autolysis by triggering murein hydrolase activity on
dividing cells (18). Although the arlRS system has been previously shown to be involved
in autolysis by negatively regulating murein hydrolase expression in an MSSA strain
such as 8325-4 (14), its contribution to autolysis by zymogram analysis in clinically
relevant MRSA strains has not been assessed. Accordingly, we performed zymogram
analysis in isogenic arlRS strains of MW2 and compared them to those of the Newman
background. As shown in Fig. 1D, there was no discernable change in the lytic profile of
various arlRS mutant as compared with the parental controls, regardless of whether
Micrococcus or S. aureus RN4220 was used as a substrate. We also deployed Newman
ΔarlRS strain as a cellular substrate in the gel to ascertain if increased autolysis in the
arlRS mutant of Newman was due to a general increase in sensitivity to autolytic
enzymes. However, these zymogram assays also showed no difference in the lytic band
profile between isogenic arlRS strains (data not shown).

Effect of mecA on Triton X-100 induced autolysis in arlRS mutants. On the basis of
similar autolytic defects between arlRS mutants of SigB-deficient RN6390 (also 8325-4)
and SigB-restored strain SH1000, we have excluded the role of sigma B as the primary
cause of enhanced autolysis mediated by arlRS (data not shown). Similarly, difference in
saeRS expression, which was found to be constitutive in Newman, but not in 6390,
SH1000 and 8325-4 (17) likely fails to explain the differences in autolysis observed
between MSSA and MRSA strains. We also tested the role of mecA by deleting the mecA
gene in the arlRS mutant of MW2 as well as expressed mecA on the xylose inducible plasmid pEPSA5 in the parental strain Newman and its isogenic arlRS mutant. In the mecA mutant of MW2, we only deleted the region encoding PBP2A in the SCCmec element. These data (Figure 2B) showed that deletion of mecA in MRSA strain MW2 did not alter the growth in Triton X-100 while addition of mecA to the arlRS mutant of MSSA strain Newman, which as expected increased its resistance to oxacillin (MIC increasing from <0.5 to 64 µg/ml), also did not restore the autolysis defect in Triton X-100. Likewise, a combined mecA and arlRS deletion in MW2 did not affect growth in medium supplemented with Triton X-100. We also evaluated the expression of pbp2 and pbp4 and did not find any significant differences in expression of these two genes between isogenic arlRS strains of Newman and MW2.

Effect of arlRS on expression of genes that regulate autolysis and murein hydrolase activity. To assess whether the Triton X-100 induced autolysis of the arlRS mutant in Newman, but not in MW2, was due to differential impacts on autolysin gene expression, Northern blots were conducted with DNA probes specific for the genes encoding major autolysins genes (atl, lytM, lytN, lytH, lytA and sle1), the TCRS lytSR and the downstream effectors lrgA which encodes a putative antiholin that interferes with the transport of murein hydrolase across the cell membrane into the cell wall (7, 8). We did not observe any differences in the transcription of genes encoding autolysins atl, lytM, lytH, lytA and sle1 between parent and the isogenic arlRS mutants. However, lytN was significantly up-regulated in the arlRS mutants of both MW2 and Newman (Fig.3A). On the contrary, the transcription of lytRS, which is a repressor of autolytic activity, and its downstream effector lrgAB was reduced in the arlRS mutants for both MW2 and
Newman (Fig. 3A). Notably, we were able to restore wild-type levels of transcription of these genes in complemented arlRS strains for both Newman and MW2 (data not shown). Given that these genes are affected in a similar manner in both MSSA Newman and MRSA MW2 backgrounds, it is unlikely that differences in transcription of these genes (lytRS and lrgA) in isogenic strains of Newman and MW2 could explain the enhanced autolysis of MSSA strain Newman in Triton X-100 but not in MRSA strain MW2. To partially confirm this, we deleted lrgAB in both arlRS mutant of Newman and MW2 and found no difference in growth under autolysis inducing conditions (data not shown). Cross complementation of the arlRS mutant of Newman in trans using a pEPSA5 based construct driving lrgAB expression also did not alleviate the Triton X-100 induced autolysis (data not shown).

Effect of arlRS deletion on expression of mgrA and agr. In our previous studies, we described mgrA as a positive regulator of agr and a negative regulator of autolysis by virtue of reduced agr and lrgAB expression in the mgrA mutant (21, 22). To ascertain whether the autolysis defect in MSSA strains was due to a differential impact of arlRS deletion on either mgrA or agr, Northern blots were conducted with DNA probes specific for mgrA, RNAII and RNAIII (Fig. 3B). Transcription of mgrA, which constitutes two overlapping transcripts from two distinct promoters, was greatly reduced in both MW2 and Newman backgrounds although the reduction is more prominent in the Newman background. The partial complementation could be the consequence of gene dosage with the plasmid pEPSA5 after induction with xylose because the same plasmid construct also led to a slight defect in Triton X-100 induced autolysis in the wild type strain. The most significant difference, however, involved transcription of both agr RNAII and RNAIII.
While a modest reduction of both transcripts was observed with the arlRS mutant of Newman, both transcripts are markedly reduced in the arlRS mutant of MW2 (Fig. 3B). To further confirm the effect of arlRS on agr expression, we conducted transcriptional fusion of the agr P3 promoter linked to the GFPuvr reporter gene. Transcriptional fusion studies revealed that agr expression is significantly down-regulated in the arlRS mutant of MW2 while the effect was more modest in the Newman background (Fig. 4). Complementation of the arlRS mutant in MW2 and Newman restored expression of RNAIII as confirmed by GFP-promoter fusion (Fig. 4) and Northern analysis (data not shown).

Effect of RNAIII over-expression on autolysis in MW2. Northern blots and promoter fusions indicated that expression of agr RNAIII was significantly down-regulated in the arlRS mutant of MW2 (Fig. 3B and 4). As agr has been described as a positive regulator of autolysis (13), we wanted to ascertain the effect of RNAIII over-expression in the arlRS mutant of MW2. For this experiment, pRN6735, which over-expressed RNAIII under a bla inducible promoter in the presence of oxacillin (Fig. 5), was introduced into MW2 and its isogenic arlRS mutant. As shown in Fig. 5, over-expression of RNAIII under oxacillin induction was able to render arlRS mutant of MW2 prone to autolysis in the presence of 0.02% Triton X-100, indicating that the down-regulation of agr in the arlRS mutant of MW2 (Fig. 3B and 4) is likely a contributing factor to its resistance in Triton X-100 mediated autolysis as compared to MSSA strain Newman. In contrast, over-expression of RNAIII in the parental strain MW2 did not have any major effect on autolysis, even under oxacillin induction. This finding indicated that, in addition to agr,
other factor(s) regulated by *arlRS* may have contributed to the propensity to autolysis in *arlRS* mutants.

Effect of *arlRS* deletion in combination with autolysins on growth and murein hydrolase activity. To assess if the increased autolysis of the *arlRS* mutant of Newman was dependent on over-expression of LytN (see Fig. 6A) or on a mechanism involving other known autolysins, in-frame deletion mutants were made for *lytN*, *atl* or *lytM* singularly and in combination with the *arlRS* mutation. All these new isogenic mutant strains grew similarly in TSB in the absence of Triton X-100 (data not shown). In the presence of 0.02% Triton X-100, however, mutation of either *atl* or *lytM* in combination with *arlRS* restored the autolytic defect while *lytN* did not (Fig. 6B). To determine the effect of these mutations on cell wall murein hydrolase activity, zymogram analysis was performed with wild-type Newman and its isogenic mutant strains. As shown in Figure 6A, an *atl* deletion with or without *arlRS* mutation, virtually abolishes all murein hydrolase activity, in line with previous studies (13, 25). A *lytM* deletion, with or without ArlRS, leads to a noticeable increase in the ~60 kDa lytic band which may correspond to the 62 kDa-amidase domain of Atl. Whether LytM might play a role in processing the Atl amidase domain or that the effect of ArlRS on LytM or Atl may be post-transcriptional remains to be determined.

Discussion

Two-component regulatory systems (TCRS) are widely used signal transduction devices that engage in a multitude of gene regulatory events in response to changing environmental conditions (4). In previous studies, it has been shown that a mutation in
the TCRS arlRS resulted in an increase in autolysis in a derivative of strain 8325-4 (14). However, we show here that a deletion in TCRS arlRS leads to enhanced autolysis in Triton X-100 only in methicillin-sensitive strains (14, 28), including Newman, SH1000, 6390 and 8325-4 (Fig. 1), but not in MRSA strains (CA-MRSA strains MW2 and USA300 and HA-MRSA strain COL) (Fig. 1). This result is surprising, considering that phylogenetically staphylococcal strains Newman and NCTC8325 are related to MRSA strains COL and USA300 (2). We have excluded sigma B or saeRS as plausible causes that account for the difference in autolysis between arlRS mutants of MSSA and MRSA strains because both sigma B positive and negative strains (RN6390, 8325, Newman, SH1000) and also strains with growth-phase dependent expression (6390, SH100 and 8325) as well as constitutive expression of saeRS (Newman) (17) all exhibited defects in autolysis in Triton X-100 in the presence of an arlRS mutation. Despite this autolytic defects, murein hydrolase activities among MW2, Newman and their arlRS isogenic mutant strain were similar. This result, combined with our finding that Triton X-100 induced autolysis in actively growing cells, may be attributable to a lack of sensitivity of our zymogram analysis assay.

We also could not attribute a lack of autolysis defect in MW2 and its isogenic arlRS mutant to mecA because the increase in autolysis was not detected in the arlRS-mecA double mutant. Furthermore, over-expression of mecA using the inducible system pEPSA5::mecA also did not rescue growth under Triton X-100 in the ΔarlRS mutant of Newman, even though mecA rendered Newman and its arlRS isogenic mutant fully oxacillin resistant (Fig. 2).
S. aureus carries several known or putative peptidoglycan hydrolases including the major autolysin atl, lytM, lytH, lytN, lytA and sle1. However, we did not find any differences in atl, lytM, lytH, lytA and sle1 transcription between the arlRS mutants and the isogenic parents. The observation that lytN transcription was up-regulated in a similar manner between the arlRS mutants and respective parents suggest that lytN is unlikely to explain the divergent propensity of the arlRS mutants to autolysis between MW2 and Newman. In addition, a lytN mutant of Newman also did not exhibit a significant increase in murein hydrolase activity as compared with the parent (see Fig. 6), again emphasizing a marginal role for lytN in the different autolytic phenotypes between these two backgrounds.

The increased rate of autolysis in arlRS mutants of MSSA strains has been linked to down-regulation of lytRS and the ensuing reduced expression of lrgAB (28), which interfere with the transport of murein hydrolase from the cytosol to the cell wall. As with lytN, both lytRS and lrgAB are down-regulated in the arlRS mutant of MW2 and Newman (Fig 3). In addition, complementation of lrgAB in trans did not positively affect growth of the arlRS mutant of strain Newman under autolytic conditions (data not shown). Together, these data suggest that lrgAB did not play a major role in autolysis in MRSA strains, in contrast to what has been found in strain 8325-4 (12).

SarA, MgrA and AgrA have also been shown to affect autolysis, with the first two regulators being a repressor of autolysis and the latter being an activator (15, 22, 46). We have found that sarA transcription was not affected in the arlRS mutant. In a previous study, we have shown that arlRS transcripts are down in an mgrA mutant (22). Here we have shown that regulation is reciprocal in that mgrA transcription is also down-
modulated in \textit{arlRS} mutants of MW2 and Newman although the difference is more prominent in the Newman background (Fig. 3). A significant difference was also seen in the \textit{agr} RNAII and RNAIII transcript levels in the $\Delta$arlRS mutant of MW2 compared to Newman \textit{$\Delta$arlRS} as assessed by Northern blot analysis and GFP-promoter fusions. Fujimoto et al. have also shown that an \textit{agr} mutant has a lower rate of autolysis rate than the wild type RN6390 strain (15), suggesting that \textit{agr} is likely a positive regulator of murein hydrolase activity. To confirm that this is also the case with MW2, we over-expressed \textit{agr} RNAIII with a plasmid containing a $\beta$-lactamase inducible promoter driving RNAIII. Our results showed that over-expression of RNAIII under oxacillin induction could render the \textit{arlRS} mutant of MW2 susceptible to hydrolysis in the presence of Triton X-100 (Fig. 5). This effect is not due to oxacillin alone since MW2, upon exposure to sub-MIC level of oxacillin did not display a dramatic increase in autolysis compared to control (Fig. 5). Nevertheless, it is clear that the dosage of RNAIII plays a crucial role in autolysis because the augmentation in autolytic activity in the \textit{arlRS} mutant of MW2 was very mild in the absence of oxacillin induction.

Besides MW2, we also ascertained if the hyper-autolytic phenotype in the \textit{arlRS} mutant of Newman could be rescued by inactivating the major autolysin genes encoding \textit{atl}, \textit{lytM}, \textit{lytN} or \textit{lytH}. Remarkably, inactivation of Atl or LytM in the \textit{arlRS} mutant of Newman was able to restore the growth pattern of the mutant comparable to that of the parental strain in 0.02\% Triton X-100 whereas a \textit{lytN} or \textit{lytH} mutation in the \textit{arlRS} mutant had no effect (Fig. 6B). Notably, an \textit{atl} deletion virtually abolished murein hydrolase activity in both wild type Newman and its isogenic \textit{arlRS} mutant while a
mutation in *lytM* led to a significant increase in a ~62 kDa band which may correspond to the Atl-derived amidase domain in both Newman and its isogenic *arlRS* mutant.

Given that many of the MSSA strains (e.g. 8325-4, SH1000) under laboratory investigations do not represent clinically-relevant isolates, our study here on the mechanisms of autolysis in CA-MRSA strain such as MW2 likely provides highly relevant information on autolysis under antibiotic inducing conditions. In particular, our data here reveal that deletion of *arlRS* in MSSA strains, but not in MRSA strains, leads to a very significant increase in Triton X-100-induced autolysis that is not attributable to alterations in the expression of sigma B, *saeRS* or *mecA*. We also found that significant down-regulation of RNAIII in MW2, which is a positive regulator of autolysis, may be part of the mechanism by which a mutation of *arlRS* in clinically relevant MRSA strains may be resistant to autolysis in the presence of a low amount of non-ionic detergent or cell-wall active antibiotics. Nevertheless, it is clear that the effect of RNAIII on autolysis is dosage dependent. In this context, it would be crucial to determine the expression of RNAIII in clinically relevant MRSA strains under antibiotic stress which can promote autolysis. The fact that over-expression of RNAIII in the wild type MW2 did not induce autolysis imply that there are additional factor(s) controlled by ArlRS that likely contributes to autolysis. In a recent study, Schlag *et al.* described the role of wall teichoic acid in targeting Atl to the staphylococcal cross-wall (42). In the absence of wall teichoic acid, Atl is evenly distributed on the cell surface, thus increasing susceptibility to autolysis. Whether *arlRS* affects the synthesis or distribution of wall teichoic acid in *S. aureus* is not defined in our studies. Nevertheless, understanding these additional genetic factors would be crucial for the development of strategies to combat MRSA.
determinants that account for this increased autolysis in MRSA strains could potentially lead to the development of novel strategies against MRSA infections.


Figure Legends.

Figure 1. Increased autolysis of arlRS mutant of strain Newman in TSB supplemented with 0.02% Triton X-100. A-B. Growth curves of MW2, Newman and their isogenic arlRS mutants without and with 0.02% Triton X-100. Overnight cultures were diluted to an OD_{650} of 0.1 in TSB and supplemented with 0.02% Triton X-100, and then grown at 37°C with shaking. The “*” indicates statistical significance in the growth of the arlRS mutant of Newman vs. the parent at all time points by the paired Student’s t-test (p<0.001). C. Map of the arlRS TCRS locus. Shown are the open reading frame designations of the MW2 and Newman genome, secondary gene names, proposed functions, translated protein sizes, and region deleted in the present study. D. Deletion of arlRS does not affect significantly the murein hydrolase activity in Newman and MW2. Zymogram analyses of cell extracts from S. aureus MW2, Newman and their isogenic arlRS mutants. Equivalent amounts of cell extracts were separated on a 8% SDS-polyacrylamide gel containing heat-killed S. aureus RN4220 cells (left panel) or Micrococcus lysodeikticus (right panel). Resolved gels were washed with water, incubated with lysis buffer (50 mM Tris, 0.1% Triton X-100, 10 mM MgCl\(_2\) and CaCl\(_2\)) for 18-24 hours and stained with 0.5% methylene blue. Areas of murein hydrolase activity are indicated by clear zones (same results were obtained with heat-killed cells of Newman and MW2 arlRS mutants).

Figure 2. The effect of complementation on autolysis of arlRS mutants of Newman and MW2. A. Complementation of arlRS on the chromosome or in trans with pEPSA5::arlRS restored wild type levels of growth in the presence of Triton X-100.
Growth curves of Newman, its isogenic arlRS mutant and complemented strains without and with 0.02% Triton X-100. The “*” indicates statistical significance in growth of arlRS mutant vs. the parent Newman at all time points by the paired Student’s t-test (p<0.001). B. The effect of mecA on autolysis of arlRS mutants of Newman and MW2. Deletion of mecA in the arlRS mutant of MW2 did not affect growth under autolysis inducing conditions while cross-complementation of the arlRS mutant of Newman had no effect on restoration of the defect in Triton X-100 induced autolysis. Growth curves of Newman wild type and arlRS mutant carrying pEPSA5::mecA, MW2 mecA and mecA/arlRS mutants in TSB 0.02% Triton X-100. The “*” indicates statistical significance of the growth the arlRS mutant vs. it isogenic parent Newman at all time points by the paired Student’s t-test (p<0.001).

Figure 3. Effect of arlRS mutation on the expression of autolysin genes and known lytic regulators. Northern blot analysis of lytN, lrgAB and lytRS (A), and mgrA, RNAII and RNAIII expression (B). Blots were hybridized with a DNA probe specific for each gene radiolabeled with [α-32P]-dCTP. Lower panels show the ethidium bromide-stained rRNAs, indicating equivalent amounts of RNA in each sample. RNAs for each strain were extracted at an OD650 of 0.7, 1.1 and 1.7 corresponding to exponential, late exponential and early stationary phases, respectively. This experiment has been repeated at least three times with similar results. A representative experiment is shown here.

Figure 4. Regulation of ArlRS on RNAIII. The expression of GFPuvr driven by the agr P3 promoter in overnight cultures. Promoter activity was plotted as mean
fluorescence/OD$_{650}$ from 3 clones in triplicates. The experiments have been repeated three times, with one set shown. The “∗” indicates statistical significance of the indicated strain as compared to MW2 by the paired Student’s t-test (p<0.001).

**Figure 5.** Over-expression of RNAIII affects growth of the arlRS mutant of MW2 under autolysis inducing conditions. Left panel: growth curves of MW2 and its isogenic arlRS mutant carrying pRN6735 with 0.02% Triton X-100. Right panel: Expression of RNAIII of arlRS mutant of MW2 with and without induction with sub-MIC concentration of oxacillin. The “∗” indicates statistical significance in OD$_{650}$nm for growth of the arlRS mutant of MW2 over-expressing agr at all time points by the paired Student’s t-test (p<0.001).

**Figure 6.** Deletion of atl or lytM affects murein hydrolase activity and restores growth in Newman arlRS mutant strain under autolysis inducing conditions. **A.** Zymogram analysis of cell extracts from *S. aureus* Newman, and its isogenic arlRS, lytN, lytM, atl or double mutants. Equivalent amounts of cell extracts were separated on an 8% SDS-polyacrylamide gel containing heat-killed *S. aureus* RN4220 cells. After electrophoresis, gels were washed with water, then buffer and finally stained with 0.5% methylene blue as described in the Procedures and Methods. Areas of murein hydrolase activity are indicated by clear zones. Arrows indicate band of increased activity in lytM and arlRS/lytM mutants. **B.** Growth curves of Newman, and its isogenic arlRS, lytN, lytM, atl or double mutants with 0.02% Triton X-100. Overnight cultures were diluted to an OD$_{650}$ of 0.1 in TSB and then supplemented with 0.02% Triton X-100, and then grown at 37°C.
with shaking. The “∗” indicates statistical significance of growth of arlRS and arlRS/lytN mutant vs. the parent Newman at all time points by the paired Student’s t-test (p<0.001).
Table 1. Strains and plasmids

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<tr>
<th>Strain or Plasmid</th>
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<td><strong>S. aureus strains</strong></td>
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<tr>
<td>RN4220</td>
<td>Heavily mutagenized MSSA strain, derivative of 8325-4, agr mutant acceptor of foreign DNA</td>
<td>(41)</td>
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<td>MW2 (USA400)</td>
<td>CA-MRSA, wild-type strain isolated in 1998 in North Dakota</td>
<td>(3)</td>
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<td>MW2 arlRS</td>
<td>ΔarlRS in-frame deletion mutant of MW2</td>
<td>This study</td>
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<td>MW2 mecA</td>
<td>ΔmecA in-frame deletion mutant of MW2</td>
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<td>ΔarlRS and ΔmecA in-frame double deletion mutant of MW2</td>
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<td>MW2 arlRS::arlRS</td>
<td>ΔarlRS complemented with arlRS with pMAD cycling</td>
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<tr>
<td>MW2 arlRS with pEPSA5::arlRS</td>
<td>ΔarlRS complemented with arlRS on pEPSA5</td>
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<td>USA300</td>
<td>CA-MRSA, wild-type strain</td>
<td>This study</td>
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<td>USA300 arlRS</td>
<td>ΔarlRS in-frame deletion mutant of USA300</td>
<td>This study</td>
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<tr>
<td>COL</td>
<td>HA-MRSA, wild-type strain isolated from a human infection in the early 1960s</td>
<td>(9, 19, 49)</td>
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<td>ΔarlRS in-frame deletion mutant of COL</td>
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<td>Newman</td>
<td>MSSA, wild-type strain isolated from a human infection in 1952</td>
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<td>Newman arlRS/lytN</td>
<td>ΔarlRS and ΔlytN in-frame double deletion mutant of Newman</td>
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<td>Newman arlRS/atl</td>
<td>ΔarlRS and Δatl in-frame double deletion mutant of Newman</td>
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<tr>
<td>Newman::mecA</td>
<td>MSSA wild-type strain expressing mecA on pEPSA5, fully methicillin resistant</td>
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Newman arlRS::mecA  \(\Delta arlRS\) of Newman expressing mecA on pEPSA5 fully methicillin resistant  This study

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<th>STRAIN</th>
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<tr>
<td>SH1000</td>
<td>MSSA, wild-type strain 8325-4 with rsbU restored</td>
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<td>SH1000 arlRS</td>
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<td>RN6390</td>
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<td>8325</td>
<td>MSSA, wild-type strain, prophage-cured strain of NCTC8325 harboring an 11-bp deletion in rsbU which regulates sigB activity by activating RsbV</td>
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<td>(\Delta arlRS) in-frame deletion mutant of strain 8325</td>
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**E. coli XL-1 blue** General lab cloning strain (40)

**Plasmids**

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<th>PLASMID</th>
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<td>pMAD</td>
<td>Allelic replacement vector to generate <em>S. aureus</em> mutant strains</td>
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<tr>
<td>pEPSA5</td>
<td>Ectopic expression vector (pEPSA5) for genes in <em>S. aureus</em></td>
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<td>pMAD arlRS-</td>
<td>pMAD containing −0.8 kb up- and downstream fragments of arlRS for deletion (DNAs from MW2 or USA300 or COL or Newman or SH1000 or 6390 or 8325)</td>
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<td>pMAD containing −0.8 kb up- and downstream MW2 fragments of mecA for deletion</td>
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<td>pMAD Newman lytM-</td>
<td>pMAD containing −0.8 kb up- and downstream Newman fragments of lytM for deletion</td>
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<td>pMAD Newman atl-</td>
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<tr>
<td>Vector</td>
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<td>pEPSA5::arlRS</td>
<td>pEPSA5 containing 2-kb DNA fragment containing the arlRS coding regions including their own rbs from MW2 or Newman at the SalI/PstI</td>
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<td>pEPSA5::mecA</td>
<td>pEPSA5 containing 2-kb DNA fragment containing the mecA coding region including its own rbs from MW2 at the BamHI/XbaI</td>
<td>This study</td>
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</table>
Figure 1

Growth Curves

- Newman WT
- Newman arlRS-
- MW2 WT
- MW2 arlRS-

Optical density (650 nm)

Time (hr)

MW1304/NWMN1233

MW1305/NWMN1234

Gene name
arlS arlR

Function
sensor kinase response regulator

Gene size
1356 bp 660 bp

Protein size
451aa 219 aa

Triton X-100 (0.02%)

Optical density (650 nm)

Time (hr)

MW2 WT
MW2 arlRS-

RN4220 cells (amidase)
M. Lysodeikticus (glucosaminidase)
Figure 3

- lytN: ∼1.2 kb
- mgrA: ∼0.6 kb, ∼0.8 kb
- lrgA: RNAIII: ∼0.5 kb, ∼1.8 kb, ∼0.8 kb
- lytS: RNAII: ∼3.5 kb

- 16S rRNA: ∼2.9 kb, ∼1.5 kb
- 23S rRNA: ∼2.9 kb, ∼1.5 kb

MW2 New
arlRS

MW2 MW2 ar
lRS- New New
ar
lRS- MW2 MW2 ar
lRS- New New
arlRS -
Figure 4

Expression of the *agr* P3-gfp reporter

Fluorescence/OD 650

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<th>MW2 ΔarlR8</th>
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<th>Newman WT</th>
<th>Newman ΔarlR8</th>
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Figure 5

Effect of RNAIII overexpression in the presence of 0.02% Triton X-100

- MW2 wt pRN6735
- MW2 wt pRN6735-oxa
- MW2 arlRS-pRN6735
- MW2 arlRS-pRN6735-oxa

Optical density (OD650)

Time (hr)

MW2 arlRS-