The *arl* locus positively regulates *Staphylococcus aureus* type 5 capsule via an *mgrA*-dependent pathway

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Most clinical *Staphylococcus aureus* strains produce either type 5 or type 8 capsular polysaccharides. The production of these capsules is influenced by various environmental factors. To study the regulation of capsule, Tn551 transposon mutagenesis and transcriptional reporter gene fusion were employed to identify several putative regulatory loci that influenced capsule gene expression. One of these, the *arl* locus, was chosen for further analysis. Tn551 was found to insert within the coding region (near the translational start site of the *arlR* gene). ArlR, along with ArlS, forms a two-component system that has been previously shown to affect autolysis and production of several secreted proteins. Phenotypic analyses of the *arlR*-specific mutant and gene fusion analyses showed that *arlR* activated capsule production at the transcriptional level. However, gel mobility shift assays did not support activation of the capsule genes by direct ArlR binding to the primary *cap5* promoter region upstream of the operon. In contrast, it was found that *arl* activated *mgrA*, an activator for capsule production, whereas *mgrA* did not have a significant effect on *arlR*. Genetic studies supported the notion that *arlR* functions upstream of *mgrA* with respect to the regulation of capsule production, although gene fusion studies indicated that *arl* could also regulate capsule independently from *mgrA*. Collectively, the results suggest that *arl* positively regulates capsule production at the transcriptional level primarily through an *mgrA*-dependent pathway.

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INTRODUCTION

Staphylococcus aureus produces several virulence factors which are essential for its pathogenicity. Among these are extracellular toxins and enzymes, and cell-wall-associated molecules. Capsular polysaccharide (CP) is one of the surface molecules produced by most *S. aureus* strains. Several serotypes of CPs have been reported to date but most human clinical *S. aureus* isolates produce capsule of either serotype 5 (CP5) or serotype 8 (CP8). The role of these capsules in *S. aureus* virulence has recently been investigated in different laboratories using molecularly characterized strains and different animal models. These studies provide compelling evidence that CP5 and CP8 are important antiphagocytic virulence factors (for reviews see Lee & Lee, 2006; O’Riordan & Lee, 2004). Additionally, staphylococcal capsule was recently implicated as one of the few extracellular factors required for virulence in *Caenorhabditis elegans* (Bae *et al.*, 2004).

The *cap5* and *cap8* operons required for the synthesis of CP5 and CP8 are allelic (Sau *et al.*, 1997a). Twelve of the 16 genes are virtually identical between the *cap5* and *cap8* operons, which explains why CP5 and CP8 have almost identical

trisaccharide-repeating units that differ only in the location of the *O*-acetylation and the position linking monosaccharides. Molecular characterization of the *cap8* locus indicates that all 16 genes are transcribed as a large transcript from the primary promoter upstream of the first gene. Several internal promoters are also present but these are much weaker than the primary promoter (Sau *et al.*, 1997b). A 10 bp inverted repeat located upstream of the –35 sequence of the primary *cap8* promoter has been shown to be required for full expression of CP8, suggesting that a DNA-binding regulator is involved in the control of capsule production (Ouyang *et al.*, 1999). The promoter regions between the *cap5* and *cap8* operons of several strains are also highly similar, suggesting that the two capsules are regulated similarly (Herbert *et al.*, 2001; Sau *et al.*, 1997a).

Several regulatory genes which globally control the expression of many virulence determinants of *S. aureus* have been identified. These regulators appear to form a complex network of regulation to coordinately control the virulence gene expression (for reviews see Bronner *et al.*, 2004; Cheung *et al.*, 2004; Novick, 2003). Previously, we showed that *agr* had a significant effect on capsule production (Luong *et al.*, 2002). Recently, we found that *mgrA*, also known as *norR* (Truong-Bolduc *et al.*, 2003) or *rat* (Ingavale *et al.*, 2003), affected capsule production (Luong *et al.*,

Abbreviation: CP, capsular polysaccharide.

2003). In this study, we employed Tn551 mutagenesis to identify several putative regulatory loci that influenced CP5/8 production. We chose to focus on *arl*, a locus which has previously been shown to regulate autolysis and some secreted proteins (Fournier & Hooper, 2000; Fournier *et al.*, 2001), and showed that the locus positively regulated CP5/8 expression at the transcriptional level mainly via an *mgrA*-dependent pathway.

METHODS

Bacterial strains and plasmids. *S. aureus* strains and plasmids used in this study are listed in Table 1. *S. aureus* strain RN4220 was used as the recipient for electroporation of the constructed plasmids. Transductions between *S. aureus* strains were carried out using bacteriophage 52A. *S. aureus* strains were cultivated on tryptic soy agar (Difco). Electroporation in *S. aureus* was carried out by the procedure of Kraemer & Iandolo (1990). Nitrocefin was purchased from Becton Dickinson Microbiology Section.

Transposon mutagenesis and identification of Tn551 insertion sites. Random transposition was carried out by temperature shift using strain COL(pI258repA36). A library of 20 000 Tn551-insertion mutants was collected and divided into 16 separate pools, and each pool was transduced with pCL8074. The transductants were then screened for increased or decreased yellow colour by spraying the colonies with 0.5 M catechol as described by Zukowski

et al. (1983). To identify Tn551 insertion sites, chromosomal DNA with Tn551 insertion was digested with *Sau*3A, ligated with T4 DNA ligase and amplified by inverse PCR using two inverse PCR primers (Tn917R and Rn917L; Table 2) as previously described (Luong *et al.*, 2003). The amplified fragments were cloned into pGEMT-easy vector (Promega) and sequenced.

Plasmid construction. Plasmid pCL96 was constructed from pCL95 (Luong & Lee, 2002) by reversing the orientation of *tet* (pT181) and *ori* (pE194) with respect to the rest of the plasmid. Plasmids pLL33 and pLL35 were constructed by ligating the *blaZ* reporter gene and the multiple cloning sites from pWN1819 and pSA3800, respectively, to pCL96. Plasmid pCL15 was derived from pSI-1 (Yansura & Henner, 1984) by removing the *Eco*RI site (end-filling), adding the ampicillin-resistance gene from pBluescript at the *Bam*HI site (which also destroyed the *Bam*HI site) and replacing the cloning site with the multiple cloning site of pUC18. To construct *Pcap5::blaZ* reporter fusions, a 627 bp fragment containing the *cap5* promoter was amplified from Newman chromosomal DNA using primers cp8bla.f and cp8bla.r. To clone the *mgrA* gene under the control of *Pspac* in pCL15, a 570 bp fragment containing the *mgrA* gene without the promoter was amplified using primers mgr74 and mgr75 and cloned into the *Hind*III and *Xba*I sites of pCL15. The same method was used to clone an 820 bp fragment containing the *arlR* gene in pCL15 using primers arlR19 and arlR20. To clone the *arlR* gene for complementation, a PCR fragment was amplified from Newman chromosomal DNA using arlR17 and arlR18 and then cloned into pCL96 to form pTL3287.

Table 1. *S. aureus* strains and plasmid vectors

Strain or plasmid	Relevant characteristics	Reference
<i>S. aureus</i>		
Becker	CP8 strain	
COL	CP5 strain	
Newman	CP5 strain	
RN4220	8325r ⁻	Kreiswirth <i>et al.</i> (1983)
CYL1164	Newman Δ <i>arlR</i>	This study
CYL815	Newman <i>arlR::Tn551</i>	This study
CYL1050	Newman Δ <i>mgrA::cat</i>	This study
CYL1206	Newman Δ <i>mgrA::cat</i> Δ <i>arlR</i>	This study
CYL1460	Newman Δ <i>mgrA</i>	This study
Plasmid		
pI258rep36	Tn551 delivery plasmid	Novick <i>et al.</i> (1979)
pCL52.2	Temperature-sensitive cloning vector	Sau <i>et al.</i> (1997b)
pCL96	Cloning vector	This study
pSA3800	Transcriptional <i>blaZ</i> fusion vector	Novick <i>et al.</i> (1989)
pWN1819	Translational <i>blaZ</i> fusion vector	Wang <i>et al.</i> (1987)
pLL33	Translational <i>blaZ</i> fusion vector	This study
pLL35	Transcriptional <i>blaZ</i> fusion vector	This study
pCL15	Cloning vector containing <i>Pspac</i> promoter	This study
pCL997	<i>Pcap5::blaZ</i> transcriptional fusion in pSA3800	This study
pCL998	<i>Pcap5::blaZ</i> translational fusion in pWN1819	This study
pAM3175	<i>Pcap5::blaZ</i> translational fusion in pLL33	This study
pAM3176	<i>Pcap5::blaZ</i> transcriptional fusion in pLL35	This study
pTL3287	pCL96 containing <i>arlR</i> gene	This study
pCL8074	<i>Pcap8::xylE</i> transcriptional fusion in pLC4	Ouyang <i>et al.</i> (1999)
pCL3292	pCL15 containing <i>mgrA</i>	This study
pTL3573	pCL15 containing <i>arlR</i>	This study

Table 2. Primers for PCR

Primer	Sequence
Tn917R	GCTATGCTCGAGTGAGTACG
Tn917L	CACAATAGAGAGATGTCACC
arlR1	CATATGACGCAAATTTTAATAG
arlR2	GGATCCTCATCGTATCACATAC
arlR3	GGTGTACATTATCCAACAGA
arlR4	AGGTGTAGATTGTTGTTGTC
arlR5	GATTGAAACAGTTCGTGGCG
arlR6	TAGATGTCAGATCCGTATCA
arlR17	CTAATTTAAAAAGCAACTATTGT
arlR18	TTACGTTTTGTCATCGTATCACAT
arlR19	ATAAAGCTTCGTAATATGAGGTGTACAAATG
arlR20	ATATCTAGAGCTCACTATTATGCAGTGTA
cp8bla.f	CTGCAGAGCTCGCATTTGAAGATCA
cp8bla.r	GGATCCCTTAGTTTGATTCACTAAA
cp8gs6	CTACTTTAGAGTATAATTATTTTAAATTC
cp8gs15	TAAAAATTTTCATTAAAAATTG
mgr74	AAGCTTTAGTATGTCGTGGAGGAGAAC
mgr75	TCTAGAACTAACAAGCTTACGCGTGC
SG16S1	CGTGCTACAATGGACAATACAAA
SG16S2	ATCTACGATTACTAGCGATTCCA
SGhu1	GCTGGGATATCAATTTCTTTACC
SGhu2	ATCCAAAACCTCACTTGCTAAAGG
SGarlR1	TTACGGTGCAGACGATTATATAG
SGarlR2	TACCGTTGACATCGATAATATCC
SGmgrA1	AGACAAGTTAATCGCTACTACTC
SGmgrA2	CTAATTCAGTTACGACTTTCTTG
SGcap8A1	ACTAAGGGTGACAATCCTCAG
SGcap8A2	AAGTCCTTTGACACCTCATCTA

Strain construction. To construct the Newman Δ arlR mutant, the upstream and downstream fragments of the target region to be replaced were amplified by PCR using two sets of primers (arlR3 and arlR4 for upstream fragment and arlR5 and arlR6 for downstream fragment) and sequence-verified. The two fragments were cloned into pCL52.2 and used for allele replacement as described previously (Lin *et al.*, 1994). The mutant (CYL1164) with a 383 bp internal deletion of the *arlR* gene without any replacing antibiotic-resistance marker was obtained and verified by PCR.

The Newman Δ mgrA::cat strain (CYL1050) was constructed by transducing Δ mgrA::cat from CYL1040 (Luong *et al.*, 2003) to strain Newman. To construct the Newman Δ mgrA strain (CYL1460), the plasmid used for the construction of CYL1040 was digested with *Apa*I to delete the *cat* gene. The resultant plasmid was transferred to CYL1050 for allele replacement as described by Lin *et al.* (1994) except that the clones were screened for the loss of chloramphenicol resistance. The resultant strain CYL1460 was confirmed by PCR with appropriate primers. To construct mgrA arlR double mutant CYL1206, the Δ mgrA::cat from CYL1040 was transduced to CYL1164.

RNA extraction and mRNA quantification. These were carried out as described previously (Luong *et al.*, 2002). For quantification of mRNA by real-time RT-PCR, gene-specific primers (SG16S1, SG16S2, SGhu1, SGhu2, SGarl1, SGarl2, SGmgrA1, SGmgrA2, SGcap8A1, SGcap8A2; Table 2) were used to amplify 100–200 nucleotide fragments of the target genes (Table 2). DNase I-treated RNA was incubated with the SuperScript III platinum SYBR Green One-Step qRT-PCR master mix (Invitrogen) and the reaction was

carried out using the ABI Prism 7300 Detection system (Applied Biosystems). No-template reactions were included as negative controls. A negative control without reverse transcriptase in the reaction was performed to exclude the possibility of DNA contamination. Standard curves were established to confirm that the primers amplified at the same rate. The 16S rRNA and/or the *hu* gene was used for normalization of the target mRNA. Relative expression levels were determined by using the protocol for the standard curve method (Applied Biosystems user bulletin no. 2).

Gel mobility shift assay. The *arlR* gene was amplified by primers arlR1 and arlR2, cloned into the *Nde*I and *Bam*HI sites of pET15b (Novagen) and used for purifying His₆-ArlR fusion protein by nickel-affinity column chromatography following the manufacturer's directions (Novagen). A 138 bp fragment containing the promoter of the *cap5* operon (from +4 to –135 with respect to the transcriptional start site) was amplified by PCR using primers cp8gs6 and cp8gs15, purified using a Qiagen PCR purification kit, end-labelled and used in gel mobility shift assay as previously described (Ouyang *et al.*, 1999). Phosphorylation of His₆-ArlR was carried out by incubating 2.3 µg (86.7 pmol) purified His₆-ArlR at 37 °C for 2 h in 10 µl reaction buffer containing 50 mM Tris/HCl (pH 8.0), 10 mM MgCl₂, 2 mM DTT and 30 mM acetyl phosphate. Successful phosphorylation was determined by detecting a shifted band in an 8 % non-denatured PAGE system.

Other tests. β -Lactamase activity was assayed by the nitrocefin method as described previously (Luong *et al.*, 2002). CP5 production was quantified as described by Luong *et al.* (2003).

Statistical analysis. Data from reporter gene fusion analyses were assessed using a paired Student's *t* test for comparing two samples. *P* values of <0.05 were considered statistically significant.

RESULTS

Screening of Tn551 mutants

To identify potential genes involved in capsule regulation, our strategy was to introduce plasmid pCL8074 carrying the *Pcap8::xylE* fusion into a mutant pool of an *S. aureus* strain and screen the colonies for mutants with altered XylE activities, which could be detected by colour change when the colonies were sprayed with the XylE substrate. A colour difference between a mutant and the wild-type would suggest that the mutation affects *cap8* gene transcription. Plasmid pCL8074 was used previously for studying *cap8* promoter activity in our laboratory (Ouyang *et al.*, 1999) but its use for monitoring the activity in isolated colonies had not been tested. We therefore transferred the plasmid into strains Becker, Newman and COL for testing XylE activity. We found that COL(pCL8074) exhibited moderate intensity of yellow colour, strain Becker(pCL8074) exhibited high intensity and Newman(pCL8074) exhibited low intensity. We chose strain COL for further transposon mutagenesis for screening potential regulatory genes because of the possibility of obtaining both classes of mutation, activator and repressor, in the same screen. Random transposition was carried out as described in Methods. Several putative mutants with reduced or enhanced yellow colour as compared to the wild-type were identified. Forty-four of these mutants were backcrossed by phage transduction to the parental strain COL and the phenotypes of 17 of them

remained unchanged, indicating that the phenotypes are due to the Tn551 insertion and not to secondary mutations during mutagenesis.

The chromosomal regions flanking Tn551 insertion sites in the 17 mutants identified above were amplified by inverse PCR, which yielded 12 different patterns according to the sizes of the amplified fragments. The fragments representing each of the 12 patterns were subjected to DNA sequencing. BLAST searches of the sequenced DNA fragment against the genomes of strains 8325 and N315 showed that the insertion sites in these mutants were within the coding region of *arlR*, *sbcD*, *sbcC*, *clpC*, N315 SA1288, N315 SA1708 and N315 SA0816. A single insertion site was found in each gene, except that there were six different insertion sites within *sbcD* gene. The insertions within *sbcC*, *sbcD* and *clpC* resulted in increased XylE activities, whereas insertions in other genes resulted in decreased XylE activities.

arlR is an activator for capsule production

To study whether the genes identified above are involved in capsule regulation, we chose to focus on *arlR*, the response regulator of the *arlRS* two-component regulatory system. The *arlRS* system has been shown to be involved in regulation of several virulence genes in *S. aureus* (Fournier & Hooper, 2000; Fournier *et al.*, 2001). The two genes form an operon, and mutations in each gene have been shown to result in the same phenotype (Fournier *et al.*, 2001). To further confirm that the *arl* locus is involved in the regulation of capsular production, we transduced the Tn551 insertion in the *arlR* gene (at between 66 and 67 bp from the translational start site) from strain COL to strain Newman, which produces type 5 capsule. The resultant strain (CYL815) produced much less capsule than the wild-type strain when the capsule was isolated from whole cell pellet or from the supernatant (Fig. 1a). We then constructed an *arlR*-deletion mutant (CYL1164) in which the 383 bp internal portion of the gene was deleted by allele replacement. As shown in Fig. 2, similar to the Tn551 mutant CYL815, the mutant CYL1164 produced an almost undetectable amount of CP5 as compared to the wild-type strain. To confirm that the mutant phenotype is due to the deletion of *arlR*, we complemented CYL1164 with pTL3287, carrying a PCR-amplified 739 bp DNA fragment containing the intact *arlR* gene from strain Newman. As shown in Fig. 2, the capsule production of the CYL1164(pTL3287) was restored to the wild-type level. Since the cloned fragment contained only the *arlR* gene without other ORFs, the results indicated that the mutation in *arlR*, not other secondary mutations or polarity, affects capsule production in strain Newman. These results suggested that the *arlR* gene, and thus the *arl* locus, is involved in the activation of capsule production.

arlR regulates *cap5* gene expression at the transcriptional level

To determine whether *arlR* regulates capsule production at the transcriptional level, we measured *cap5*-specific mRNA

Fig. 1. Comparison of strains Newman and CYL815. (a) CP5 production. Equivalent amounts of samples adjusted according to OD₆₆₀ were taken from overnight cultures (at OD₆₆₀ 7.5 and 7.7 for Newman and CYL815, respectively) and processed as described in Methods. Samples were diluted threefold initially and further threefold serially diluted. The diluted samples (20 µl samples from pellet and 252 µl samples from supernatant) were loaded to nitrocellulose membranes and incubated with rabbit anti-CP5 specific antibody. Threefold dilutions of CP5 standard with an initial content of 6.7 ng in 20 µl were loaded as controls. (b) *cap5D*-specific mRNA. Total RNAs were purified from overnight cultures. The undiluted (10 µg) and the twofold serially diluted samples were loaded to nitrocellulose membranes and probed with the *cap5D* gene probe (top panel). A duplicate set of samples was probed with the *S. aureus* 16S rRNA gene (bottom panel) to indicate equal loading of the samples.

synthesis in the wild-type strain Newman and its isogenic *arlR* mutant CYL815 by RNA slot-blotting. Total RNAs were isolated from overnight cultures of Newman and CYL815 and hybridized either with the *cap5D* gene probe or with a 16S rRNA gene probe as a loading control. The results in Fig. 1(b) show that the amount of *cap5* mRNA isolated from the mutant CYL815 was much less than that isolated from Newman. This result is similar to the assay of CP5 production described above, suggesting that *arlR* is an activator regulating CP5 production primarily at the transcriptional level.

To confirm the transcriptional regulation of *cap5* expression by *arlR* and to determine whether *arlR* can also have an

Fig. 2. CP5 from strains Newman, CYL1164 and the complemented strain CYL1164(pTL3287) from overnight cultures (OD_{660} 7.3, 6.9 and 7.0, respectively) was isolated as described in Methods. The undiluted (30 μ l) and the threefold serially diluted samples (indicated above the blot) were analysed by Western dot-blotting.

effect on CP5 production at the post-translational level, we constructed *blaZ* reporter gene fusions. The fusion plasmids were transduced into strain Newman and mutant strain CYL1164. As shown in Fig. 3, the *Pcap5* activity in the *arlR* mutant was significantly less than that in the wild-type strain in both transcriptional and translational fusion assays at all time points. We also obtained similar results for *BlaZ* activities when the fusion plasmids were put into the Tn551 insertional mutants (data not shown). The fact that the *BlaZ* activities decreased in a similar pattern in both transcriptional and translational fusions suggests that the regulation of the *cap5* genes by *arlR* is controlled at the transcriptional level. However, we noted that translational fusions had a tenfold higher activity than the transcriptional fusions (Fig. 3). This difference could be due to the difference in ribosome-binding site of the two constructs.

ArlR activates *cap5* promoter indirectly

To determine whether ArlR activates the *cap5* genes by direct binding, we performed gel mobility shift experiments by incubating a 32 P-labelled DNA fragment of 138 bp from the promoter region of the *cap5* locus containing the 10 bp inverted repeat previously shown to be required for *cap5* activation (Ouyang *et al.*, 1999) with increasing concentrations of purified His₆-ArlR. We found a shifted band, which could be inhibited with 500-fold excess of unlabelled competitor DNA but not by an irrelevant DNA fragment (data not shown). However, the shifting could only be detected when the amount of ArlR was or exceeded 5 μ M, suggesting that ArlR binds to the *cap5* promoter region but only at a high concentration. Phosphorylation of ArlR with acetyl phosphate did not facilitate the binding (data not shown). Taking these results together, we concluded that ArlR might not regulate the capsule production by direct binding to the promoter region of the *cap5* operon. Although the binding occurred under high concentrations of the protein, it was most likely not physiologically relevant.

arlR regulates *cap5* genes primarily through *mgrA*

We have previously shown that *mgrA* is a major activator of *cap5(8)* gene expression (Luong *et al.*, 2003, 2006). Since we showed above that *arl* most likely did not regulate the *cap5* genes directly, we speculated that it might regulate capsule production through *mgrA*. To test this possibility, we compared the *mgrA* mRNA produced from wild-type strain Newman and an *arl* deletion mutant from 18 h cultures using real-time RT-PCR. We found that the *mgrA* mRNA was reduced 18-fold in the *arlR* mutant (data not shown), indicating that *mgrA* is positively regulated by the *arl* locus. On the other hand, the amount of *arlR* mRNA from an *mgrA* mutant was similar to that from the wild-type, indicating

Fig. 3. *BlaZ* activities of *cap5::blaZ* transcriptional fusion in plasmid pCL997 (a) and of *cap5::blaZ* translational fusion in plasmid pCL998 (b) in Newman and CYL1164 were analysed at 2, 4, 6, 8 and 24 h. The *BlaZ* activities are expressed as the ratio of A_{482} of the enzymic reaction to the OD_{660} of the culture. The error bars indicate SD ($n=3$).

Fig. 4. Comparison of *cap5* gene expression in $\Delta mgrA$ [CYL1640(pCL15)], $\Delta mgrA + mgrA$ [CYL1640(pCL3292)] and $\Delta mgrA + arlR$ [CYL1640(pTL3573)] (a), and in $\Delta arlR$ [CYL1164(pCL15)], $\Delta arlR + mgrA$ [CYL1164(pCL3292)] and $\Delta arlR + arlR$ [CYL1164(pTL3573)] (b). Total RNAs were isolated from 18 h cultures in the presence of 1 mM IPTG; the *cap5* mRNAs were quantified by real-time RT-PCR and shown as a ratio to that of strain Newman (arbitrarily set at 1). The error bars indicate SD (n=3).

that *mgrA* has no significant effect on *arl* expression. These results suggest that *arl* may up-regulate *cap5* through up-regulation of *mgrA*.

To further confirm that *arl* functions upstream of *mgrA* with respect to *cap5* gene regulation, we measured the *cap5* mRNA by real-time RT-PCR to determine whether over-producing MgrA can compensate *cap5* expression in an *arlR* mutant and, conversely, whether overproducing ArlR can compensate *cap5* expression in an *mgrA* mutant. The rationale for these experiments is that overproduction of MgrA, but not overproduction of ArlR, should restore the *arl* effect on capsule if *arl* is upstream of *mgrA* in the regulatory circuit. On the other hand, a reverse effect should be detected if *arl* acts downstream of *mgrA*. To this end, we cloned the *mgrA* or *arlR* gene under the control of the IPTG-inducible promoter *Pspac* in pCL15. The results in Fig. 4(a) show that MgrA overproduction was able to restore the

cap5 mRNA in the *arl* mutant to the wild-type level, whereas the results in Fig. 4(b) show that ArlR overproduction did not restore the *cap5* mRNA level in the *mgrA* mutant. These results support the contention that *arl* functions upstream of the *mgrA*.

To determine whether *arl* regulates the *cap5* genes solely through *mgrA*, we compared the transcriptional and translational *blaZ* reporter activities in Newman and the isogenic *arl*, *mgrA* and double *arlR mgrA* mutants. Since the *mgrA arlR* double mutant strain CYL1206 was constructed with a chloramphenicol-resistance marker, the above *Pcap5::blaZ* fusion plasmids pCL997 and pCL998, which have the same resistance marker, cannot be used. We therefore reconstructed the *Pcap5::blaZ* fusions in tetracycline-resistant pCL33 and pLL35 vectors, which resulted in transcriptional fusion plasmid pAM3175 and translational fusion plasmid pAM3176, respectively. As shown in Fig. 5,

Fig. 5. The activities of *cap5::blaZ* transcriptional fusion in plasmid pAM3176 (a) and translational fusion in plasmid pAM3175 (b) in strains Newman, CYL1050 (Newman $\Delta mgrA::cat$), CYL1164 (Newman $\Delta arlR$) and CYL1206 (Newman $\Delta mgrA::cat$ $\Delta arlR$) were analysed at 4, 6, 8 and 24 h. The *BlaZ* activities were expressed as the ratio of A_{482} of the enzymic reaction to the OD_{660} of the culture. The error bars indicate SD (n=3).

for both transcriptional and translation fusions, mutations in *mgrA* or *arlR* substantially reduced the *cap5* promoter activity, and the double mutation reduced the activity more than the single mutations. The additional reduction in *cap5* promoter activities at various time points in the double mutant as compared to that of the *mgrA* single mutant are statistically significant, with *P* values (paired *t* test) ranging from 0.0005 to 0.0135 for the transcriptional *blaZ* assays (Fig. 5a) and from 0.0029 to 0.021 for the translational *blaZ* assays (Fig. 5b). This reduction of the *cap5* promoter activity resulting from *arl* mutation in the *mgrA* mutant suggests that *arl* also regulates *cap5* independently from *mgrA*. However, although the reduction is statistically significant, it is only moderate, suggesting that the *mgrA*-independent pathway(s) may only play a minor role in *cap5* regulation.

DISCUSSION

S. aureus can cause a variety of diseases. Its pathogenicity depends on coordinated expression of various virulence genes. Regulation of these virulence genes is therefore critical in determining the outcome of an infection. In this study, we used transposon mutagenesis to generate random mutations in strain COL and identified several potential regulators that affect the production of capsule, one of the important virulence factors produced by most *S. aureus* strains. We identified several potential genes affecting capsule production. However, some known regulatory genes involved in capsule regulation, such as *agr* or *mgr*, were not identified, suggesting that the library is biased to some degree. Among the genes identified, we chose to focus on *arl* since this locus has been shown to be an important regulator affecting the expression of several virulence factors in *S. aureus*, including autolysin and various toxins (Fournier & Hooper, 2000; Fournier *et al.*, 2001), but how it regulates capsule has not been studied. Our results showed that *arl* positively controlled the production of capsule. These results are consistent with a recent gene profiling study showing that two of the *cap5* genes were upregulated by *arl* (Liang *et al.*, 2005) and with a study showing that a natural deletion in the *arlRS* locus of the sequenced N315 strain affected CP5 production (Cocchiario *et al.*, 2006). The *arl* locus is composed of two genes, *arlS* and *arlR*, encoding the sensor and regulator, respectively, of a typical prokaryotic two-component regulatory system in which the sensor senses an environmental cue(s) and relates the information to the response regulator, usually, by phosphorylation (Klumpp & Kriegstein, 2002). However, to date, no environmental cue has been reported to be mediated by ArlS. Several environmental factors, which include iron concentration, oxygen tension, carbon dioxide and growth media, have been reported to influence capsule production (Lee & Lee, 2006; O'Riordan & Lee, 2004). The environmental control of virulence gene expression in *S. aureus* is an understudied area. It would be of interest to determine if the signal of any of these environmental factors affecting capsule production is transduced through the *arl* system.

The expression of virulence genes in *S. aureus* is controlled by very complicated regulatory circuitry (Bronner *et al.*, 2004; Cheung *et al.*, 2004; Novick, 2003) in which various regulators form a complex network to coordinately regulate different virulence genes. With regard to capsule, we have previously shown that both *agr* and *mgr* positively regulate capsule production mostly at the transcriptional level, whereas *sarA* does not play a role in transcriptional regulation but may have a minor effect at the post-translational level (Luong *et al.*, 2002, 2003). In this study, we found that *arl* played an activator role, exerting its effect at the transcriptional level, similar to that of *agr* and *mgr*. However, how *arl* regulates its target genes has not been studied. Since *arl* is a two-component system, it is likely that ArlR binds some target genes directly. Although our gel mobility shift experiments showed that both the unphosphorylated and phosphorylated forms of ArlR could bind to the *cap5* promoter region, the concentrations of the protein at which the binding occurred were most likely too high to be within the physiological condition. Hence the binding may not be biologically significant. Our results, therefore, suggest that ArlR most likely does not regulate capsule production by direct binding to the *cap5* promoter region.

As the gel-shift experiments indicated that *arl* probably regulates capsule through other regulators, to test this possibility we investigated whether *arl* affected *mgrA* expression. We found that *mgrA* expression was drastically reduced (18-fold) in an *arlR* deletion mutant, whereas *mgrA* mutation did not affect the expression of *arlR*, indicating that *arl* activates *mgrA* but not the opposite. We also showed that *mgrA* expressed from an inducible promoter could override the *arl* effect on *cap5* genes but not vice versa, suggesting that *arl* functions upstream of *mgrA* in activating capsule. These results therefore strongly indicate that *arlR* activates *cap5* genes through *mgrA*. Interestingly, further experiments using gel-shift assays did not find evidence of ArlR binding to the *mgrA* promoter or MgrA binding to the *cap5* promoter (results not shown), suggesting that intermediary regulators exist upstream and downstream of *mgrA* in the *arlRS-mgrA-cap5* regulatory pathway.

It should be noted here that our quantitative RT-PCR results showing that *mgrA* did not affect *arl* expression are inconsistent with those reported from Ingavale *et al.* (2003) and Manna *et al.* (2004). In these two previous reports, MgrA was first shown to activate *arl* expression by direct binding to the *arl* promoter region (Ingavale *et al.*, 2003) but was later reported to indirectly repress *arl* through *sarV* (Manna *et al.*, 2004). Because these two studies used the same strain (the *sigB*-deficient RN6390) and the results are contradictory, it is difficult to draw a firm conclusion as to how *mgrA* regulates *arl* based on the studies. On the other hand, since the *sigB*-positive strain Newman was used in our studies, it is conceivable that the discrepancy between our results and those from the two reports could be explained by strain difference.

In this study, besides *arl*, we also identified five additional potential regulatory loci affecting capsule gene expression.

Our preliminary data showed that two of these newly identified regulators, *sbcDC* and *clpC*, were also involved in capsule gene regulation. In addition to *mgrA* and *agr*, recent reports showed that *sae* and *sigB* were also involved in capsule regulation (Steinhuber *et al.*, 2003; Bischoff *et al.*, 2004). Taken together, these results indicate that capsule is under the regulation of a surprisingly large number of regulators. One interesting question to ask would be why capsule regulation is regulated by so many regulators. Capsular polysaccharide is a large molecule that resides on the surface of the bacteria. Because of its size, capsule anchored on the cell surface is an important defence shield for the bacteria to avoid host phagocytosis by masking the C3b deposited on the bacterial cell wall from the phagocytes (Cunnion *et al.*, 2003; Karakawa *et al.*, 1988; Thakker *et al.*, 1998). However, for the same reason, it can also mask other surface components required for staphylococcal pathogenesis. Indeed, it has been shown that capsule impedes the initial attachment of *S. aureus* to endothelial cells by masking the adhesins (Pohlmann-Dietze, 2000). Thus, the organism needs to regulate capsule production according to requirements at various stages of the infection process. *S. aureus* can cause various types of infection at various sites of the host; it is therefore not unreasonable to postulate that capsule is regulated differently in different infections. The need for the organism to fine-tune capsule production under different conditions may explain why capsule is regulated by several regulators. The impressive number of regulatory genes involved in capsule regulation further supports the notion that virulence genes in *S. aureus* are regulated by a complex regulatory network.

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REFERENCES

- Bae, T., Banger, A. K., Wallace, A., Glass, E. M., Aslund, F., Schneewind, O. & Missiakas, D. M. (2004). *Staphylococcus aureus* virulence genes identified by *bursa aurealis* mutagenesis and nematode killing. *Proc Natl Acad Sci U S A* **101**, 12312–12317.
- Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bächi, B. & Projan, S. (2004). Microarray-based analysis of the *Staphylococcus aureus* σ^B regulons. *J Bacteriol* **186**, 4085–4099.
- Bronner, S., Monteil, H. & Prevost, G. (2004). Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. *FEMS Microbiol Rev* **28**, 183–200.
- Cheung, A. L., Bayer, A. S., Zhang, G., Gresham, H. & Xiong, Y. Q. (2004). Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunol Med Microbiol* **40**, 1–9.
- Cocchiario, J. L., Gomez, M. I., Risley, A., Solinga, R., Sordelli, D. O. & Lee, J. C. (2006). Molecular characterization of the capsule locus from non-typeable *Staphylococcus aureus*. *Mol Microbiol* **59**, 948–960.
- Cunnion, K. M., Zhang, H. M. & Frank, M. M. (2003). Availability of complement bound to *Staphylococcus aureus* to interact with membrane complement receptors influences efficiency of phagocytosis. *Infect Immun* **71**, 656–662.
- Fournier, B. & Hooper, D. C. (2000). A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. *J Bacteriol* **182**, 3955–3964.
- Fournier, B., Klier, A. & Rapoport, G. (2001). The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. *Mol Microbiol* **41**, 247–261.
- Herbert, S., Newell, S. W., Lee, C. Y., Wieland, K. P., Dassy, B., Fournier, J. M., Wolz, C. & Döring, G. (2001). Regulation of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides by CO₂. *J Bacteriol* **183**, 4609–4613.
- Ingavale, S. S., Van Wamel, W. & Cheung, A. L. (2003). Characterization of RAT, an autolysis regulator in *Staphylococcus aureus*. *Mol Microbiol* **48**, 1451–1466.
- Karakawa, W. W., Sutton, A., Schneerson, R., Karpas, A. & Vann, W. F. (1988). Capsular antibodies induce type-specific phagocytosis of capsulated *Staphylococcus aureus* by human polymorphonuclear leukocytes. *Infect Immun* **56**, 1090–1095.
- Klumpp, S. & Krieglstein, J. (2002). Phosphorylation and dephosphorylation of histidine residues in proteins. *Eur J Biochem* **269**, 1067–1071.
- Kraemer, G. R. & Iandolo, J. J. (1990). High-frequency transformation of *Staphylococcus aureus* by electroporation. *Curr Microbiol* **21**, 373–376.
- Kreiswirth, B. N., Lofdahl, S., Betley, M. J., O'Reilly, M., Shlievert, P. M., Bergdoll, M. S. & Novick, R. P. (1983). The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* **305**, 709–712.
- Lee, C. Y. & Lee, J. C. (2006). Staphylococcal capsules. In *Gram-Positive Pathogens*, 2nd edn, pp. 456–463. Edited by V. Fischetti, R. P. Novick, J. Ferretti, D. Portnoy & J. Rood. Washington, DC: American Society for Microbiology.
- Liang, X., Zheng, L., Landwehr, C., Lunsford, D., Holmes, D. & Ji, Y. (2005). Global regulation of gene expression by ArlRS, a two-component signal transduction regulatory system of *Staphylococcus aureus*. *J Bacteriol* **187**, 5486–5492.
- Lin, W. S., Cunneen, T. & Lee, C. Y. (1994). Sequence analysis and molecular characterization of genes required for the biosynthesis of type 1 capsular polysaccharide in *Staphylococcus aureus*. *J Bacteriol* **176**, 7005–7016.
- Luong, T. T. & Lee, C. Y. (2002). Overproduction of type 8 capsular polysaccharide augments *Staphylococcus aureus* virulence. *Infect Immun* **70**, 3389–3395.
- Luong, T. T., Sau, S., Gomez, M., Lee, J. C. & Lee, C. Y. (2002). Regulation of *Staphylococcus aureus* capsular polysaccharide expression by *agr* and *sarA*. *Infect Immun* **70**, 444–450.
- Luong, T. T., Newell, S. W. & Lee, C. Y. (2003). *mgr*, a novel global regulator in *Staphylococcus aureus*. *J Bacteriol* **185**, 3703–3710.
- Luong, T. Y., Dunman, P. M., Murphy, E., Projan, S. J. & Lee, C. Y. (2006). Transcriptional profiling of the *mgrA* regulon in *Staphylococcus aureus*. *J Bacteriol* **188**, 1899–1910.
- Manna, A. C., Ingavale, S. S., Maloney, M., van Wamel, W. & Cheung, A. L. (2004). Identification of *sarV* (SA2062), a new transcriptional regulator, is repressed by SarA and MgrA (SA0641) and involved in the regulation of autolysis in *Staphylococcus aureus*. *J Bacteriol* **186**, 5267–5280.
- Novick, R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* **48**, 1429–1449.

- Novick, R. P., Edelman, I., Schwesinger, M. D., Gruss, A. D., Swanson, E. C. & Pattee, P. A. (1979). Genetic translocation in *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **76**, 400–404.
- Novick, R. P., Iordanescu, S., Projan, S. J., Kornblum, J. & Edelman, I. (1989). pT181 plasmid replication is regulated by a countertranscript-driven transcriptional attenuator. *Cell* **59**, 395–404.
- O’Riordan, K. & Lee, J. C. (2004). *Staphylococcus aureus* capsular polysaccharides. *Clin Microbiol Rev* **17**, 218–234.
- Ouyang, S., Sau, S. & Lee, C. Y. (1999). Promoter analysis of the *cap8* operon involved in type 8 capsular polysaccharide production in *Staphylococcus aureus*. *J Bacteriol* **181**, 2492–2500.
- Pohlmann-Dietze, P., Ulrich, M., Kiser, K. B., Doring, G., Lee, J. C., Fournier, J. M., Botzenhart, K. & Wolz, C. (2000). Adherence of *Staphylococcus aureus* to endothelial cells: influence of the capsular polysaccharide, the global regulator *agr*, and the bacterial growth phase. *Infect Immun* **68**, 4865–4871.
- Sau, S., Bhasin, N., Wann, E. R., Lee, J. C., Foster, T. J. & Lee, C. Y. (1997a). The *Staphylococcus aureus* allelic genetic loci for serotype 5 and 8 capsule expression contain the type-specific genes flanked by common genes. *Microbiology* **143**, 2395–2405.
- Sau, S., Sun, J. & Lee, C. Y. (1997b). Molecular characterization and transcriptional analysis of type 8 genes in *Staphylococcus aureus*. *J Bacteriol* **179**, 1614–1621.
- Steinhuber, A., Goerke, C., Bayer, M. G., Doring, G. & Wolz, C. (2003). Molecular architecture of the regulatory locus *sae* of *Staphylococcus aureus* and its impact on expression of virulence factors. *J Bacteriol* **185**, 6278–6286.
- Thakker, M., Park, J.-S., Carey, V. & Lee, J. C. (1998). *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. *Infect Immun* **66**, 5183–5189.
- Truong-Bolduc, Q. C., Zhang, X. & Hooper, D. C. (2003). Characterization of NorR protein, a multifunctional regulator of *norA* expression in *Staphylococcus aureus*. *J Bacteriol* **185**, 3127–3138.
- Wang, P. Z., Projan, S. J., Leason, K. R. & Novick, R. P. (1987). Translational fusion with a secretory enzyme as an indicator. *J Bacteriol* **169**, 3082–3087.
- Yansura, D. G. & Henner, D. J. (1984). Use of *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **81**, 439–443.
- Zukowski, M. M., Gaffiney, D. G., Speck, D., Kauffmann, M., Findeli, A., Wisecup, A. & Lecocq, J. P. (1983). Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned *Pseudomonas* gene. *Proc Natl Acad Sci U S A* **80**, 1101–1105.