Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Antisense locked nucleic acids targeting *agrA* inhibit quorum sensing and pathogenesis of community-associated methicillin-resistant *Staphylococcus aureus*

F. Da[1,*], L. Yao[2,*], Z. Su[1], Z. Hou[1], Z. Li[1], X. Xue[1], J. Meng[1] and X. Luo[1]

1 Department of Pharmacology, School of Pharmacy, Fourth Military Medical University, Xi'an, China
2 Department of Medicinal Chemistry, School of Pharmacy, Fourth Military Medical University, Xi'an, China

Keywords
agr, antisense, locked nucleic acid. methicillin-resistant *Staphylococcus aureus*, skin infection, virulence.

Correspondence
Jingru Meng and Xiaoxing Luo, Department of Pharmacology, School of Pharmacy, Fourth Military Medical University, Xi'an 710032, China.
E-mails: mjrfmmu@fmmu.edu.cn and xxluo3@fmmu.edu.cn

*F.D. and L.Y. contributed equally to this work.

2016/2038: received 20 May 2016, revised 16 September 2016 and accepted 3 October 2016

doi:10.1111/jam.13321

Abstract

Aim: Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is commonly associated with nonnosocomial skin and soft tissue infections due to its virulence, which is mainly controlled by the accessory gene regulator (*agr*) quorum sensing (QS) system. In this study $(KFF)_3K$ peptide-conjugated locked nucleic acids (PLNAs) targeting *agrA* mRNA were developed to inhibit *agr* activity and arrest the pathogenicity of CA-MRSA.
Methods and Results: Two PLNAs were designed, and synthesized, after predicting the secondary structure of *agrA* mRNA. The influence on bacterial growth was tested using a growth curve assay. RT-qPCR, haemolysis assay, lactate dehydrogenase release assay and chemotaxis assay were used to evaluate the effects of the PLNAs on inhibiting *agr* QS. A mouse skin infection model was employed to test the protective effect of the PLNAs *in vivo*. None of the PLNAs were found to be bacteriostatic or bactericidal *in vitro*. However, one PLNA, PLNA34, showed strong ability to suppress expression of *agrA* and the effector molecule *RNAIII* in USA300 LAC strain. Furthermore, PLNA34 inhibited the expression of virulence genes that are upregulated by *agr*, including *hla*, *psmα*, *psmβ* and *pvl*. The haemolytic activity of the supernatants from PLNA34-treated bacteria was also dramatically reduced, as well as the capacity to lyse and recruit neutrophils. Moreover, PLNA34 showed high levels of protection in the CA-MRSA mouse skin infection model.
Conclusions: The anti-*agrA* PLNA34 can effectively inhibit the *agr* QS and suppress CA-MRSA pathogenicity.
Significance and Impact of the Study: *agrA* is a promising target for the development of antisense oligonucleotides to block *agr* QS.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is still a major cause of hospital-, community- and livestock-associated infections, posing a significant threat to human health (Chambers and Deleo 2009). In many countries, MRSA rates have reached or exceeded 50% among *S. aureus* isolates from infections (Diekema *et al.* 2001). In the US alone, more than 80 000 invasive MRSA infections and 11 000 related deaths occur annually (CDC 2013). Compared with hospital-associated (HA-) MRSA, community-associated (CA-) MRSA is more virulent and can cause disease in otherwise healthy individuals (Otto 2013). The rapid emergence of resistance to most available antibiotics in recent years has severely complicated MRSA therapy (Moellering 2012). Traditional antibiotics have either bacteriostatic or bactericidal activity, imposing selective pressure on bacterial survival and inevitably leading to drug resistance. Therefore, novel alternative strategies to combat MRSA infections are currently being developed, including strategies that target virulence or quorum sensing (QS) systems (Khan *et al.* 2015).

Quorum sensing, an important bacterial regulatory system that relies on the detection of extracellular autoinducers, have been shown to control virulence in many bacterial pathogens (Reuter *et al.* 2016). For *S. aureus*, the accessory gene regulator (*agr*) QS is the predominant and best-studied virulence regulator and is responsible for increased virulence gene expression, including many toxins and degradative exoenzymes, that are crucial for establishment of infection (Le and Otto 2015). Higher *agr* activity is also thought to be responsible for the higher virulence of CA-MRSA than HA-MRSA (Cheung *et al.* 2011). Recently, mouse and rabbit infection models have demonstrated that *agr* knock-out strains are less pathogenic than wild-type strains (Cheung *et al.* 2011; Kobayashi *et al.* 2011). In this regard, *agr*-targeted therapeutic strategies are an attractive proposition.

The *agr* locus is encoded by *RNAII* and *RNAIII*, which are divergently transcribed from the P2 and P3 promoters respectively. *RNAII* consists of four genes, *agrB, agrD, agrC* and *agrA*. AgrD is a peptide precursor that is processed and exported by AgrB on the membrane, forming the extracellular QS signal called autoinducing peptide (AIP). The sensor histidine kinase AgrC and transcription factor AgrA constitute a classical two-component signalling system (TCS) that respond to the presence of AIP. *RNAIII*, the effector molecule, is a small regulatory RNA that can upregulate the expression of toxin genes related to infection, such as *hla* and *pvl* (Queck *et al.* 2008). Once a critical concentration is reached, AIP binds to and activates AgrC, leading to phosphorylation of AgrA, which then binds to the P2 and P3 promoters, inducing *RNAIII* transcription and subsequent amplification of the AIP signal. In addition, phosphorylated AgrA directly binds to the promotors of the PSMα and PSMβ peptides, toxins that also play an important role in the pathogenicity of CA-MRSA (Queck *et al.* 2008).

To date, four different AIPs (AIP I–IV), with varying levels of sequence homology, have been identified in *S. aureus*. Each AIP specifically activates its cognate AgrC receptor while antagonizing receptors from other groups. Therefore, the majority of studies aimed at disrupting *agr* have been mainly focused on blocking AIP binding to its cognate AgrC receptor using modified or heterogenous AIP, or monoclonal antibodies against AIP (Wright *et al.* 2005; Park *et al.* 2007; Tal-Gan *et al.* 2013). However, the limitation of this strategy, especially using antibodies, is that is cannot target all four *agr* types. AgrA is relatively conserved compared to the variability of AIPs and AgrC, which, in addition to its critical role in *agr*, suggests that it may be an effective drug target to inhibit *agr* QS. Recently, small chemical molecules designed to block AgrA phosphorylation or inhibit the AgrA–DNA interaction have been reported. However, most of these compounds, with the exception of savarin, have not been tested *in vivo*. So far, savarin is the most promising small molecule inhibitor that disrupts *agr* QS *in vitro* and *in vivo* by blocking AgrA–DNA binding in *S. aureus* (Sully *et al.* 2014). Here, we provide another strategy, antisense oligonucleotides, to inhibit AgrA expression at the mRNA level. Our results show that *agr* QS can be effectively quenched by peptide-locked nucleic acid LNAs conjugates that target *agrA* mRNA.

Materials and methods

Bacterial strains and growth conditions

CA-MRSA USA300 LAC (Los Angeles Clone) strain was grown in tryptone soy broth (TSB) (Oxoid, UK) overnight at 37°C with shaking at 180 rev min^{-1}.

Design and synthesis of locked nucleic acids

The sequence of *agrA* mRNA was obtained from the Genbank Nucleotide database, and homologues of *agrA* gene coding regions among staphylococcal strains were identified by BLAST. Primer Premier 5.0 was used to predict oligonucleotides (18–24 bp) that would bind to *agrA* mRNA. Secondary structure of *agrA* mRNA and free energy (ΔG) of complementary antisense oligonucleotides were predicted by RNA structure 4.5 software and targets that met the requirements (overall $Tm \geq 50$°C, GC % = 40–60%, $\Delta G < -10$ kcal per mol, Duplex $\Delta G < -25$ kcal per mol, oligo-self $\Delta G \geq -1{\cdot}1$ kcal per mol, oligo-oligo $\Delta G \geq -8$ kcal per mol (Matveeva *et al.* 2003)), were selected. The selected oligonucleotide sequences were synthesized as LNAs and covalently conjugated to the cell penetrating peptide $(KFF)_3K$, using a cys–succinimidyl*trans*-4-(maleimidylmethyl)cyclohexane-1-carboxylate-C6 (Cys-SMCC-C6) linker to give cell penetrating peptide-conjugated LNAs (PLNAs). All PLNAs were synthesized and purified by Bio-Synthesis Inc. (Lewisville, TX, USA), and stored in ddH_2O at −80°C.

Bacterial growth assay

To determine the inhibitory effect of PLNAs on *S. aureus* LAC, overnight cultures were diluted to an $OD_{630\ nm}$ of 0·1 (equivalent to 1×10^8 colony-forming unit (CFU) per ml) in 50 μl TSB in a 96-well microtitre plate. Stocks of PLNA34 and PLNA522 were added immediately to a final concentration of 12·5, 25 and 40 μmol l^{-1} each in a total volume of 100 μl. Phosphate buffered saline (PBS) was added to LAC and used as the blank control. The growth rate of the cells was determined by measuring the $OD_{630\ nm}$ values with a microplate reader (Bio-Rad

Laboratories, Tokyo, Japan) at different time points (0, 1, 2, 3, 4 and 5 h).

RT-qPCR analysis

LAC (50 μl) in TSB (1×10^8 CFU per ml) was grown with PLNA34, PLNA522 or scrambled PLNA34 for 5 h at 37°C in a total volume of 100 μl. The concentration of PLNAs was 3·125, 6·25 or 12·5 μmol l^{-1}. LAC was incubated with PBS as a control. After 5 h of incubation, cell pellets were harvested by centrifugation and RNA was extracted using RNeasy columns (Qiagen China Co. Ltd, Shanghai, China) according to manufacturer's protocol. Total RNA (2 μl of 0·5 μg μl^{-1}) was used for each reverse transcription reaction in a 20 μl final volume with PrimeScript RT reagent Kit with gDNA Eraser (Takara Biotechnology Co. Ltd, Dalian, China). The resulting cDNA was stored at −80°C before use. The *16S rRNA*, *agrA*, *RNAIII*, *hla*, *psmα*, *psmβ* and *pvl* genes were amplified in 25 μl reactions that contained 12·5 μl 2× SYBR Premier ExTaq™ (Takara Biotechnology Co. Ltd, Dalian, China), 2 μl template (1 : 20 diluted cDNA), 1 μl for each primer (10 μmol l^{-1}) and 8·5 μl sterile distilled water. Thermal cycling conditions: step 1: 95°C, 10 min; step 2: 95°C, 5 s, 55°C, 30 s, 40 cycles; step 3: 95°C, 15 s, 55°C, 30 s, 95°C, 15 s. PCR amplification was performed using the Stratagene Mx3000P QPCR System (Genetimes Technology, Inc., Shanghai, China). All PCR reactions were performed in triplicate. Primer sequences are shown in Table 1. PCR efficiencies were included in all analyses. *16S rRNA*, which exhibited no change in expression in all tested samples (see Fig. S1), served as reference gene to normalize changes in transcription levels between samples. Relative expression levels were determined by the $2^{-\Delta\Delta Cq}$ method (Winer *et al.* 1999). Briefly, for each sample, the difference of quantification cycle (ΔCq) was calculated by $\Delta Cq = Cq_{(\text{target gene})} - Cq_{(16S\ rRNA)}$. The ΔΔCq was obtained from $\Delta\Delta Cq = \Delta Cq_{(\text{PLNAs group})} - \Delta Cq_{(\text{PBS group})}$; then, relative expression = $2^{-\Delta\Delta Cq}$.

Haemolysis assay

The relative haemolytic activity of culture supernatants was evaluated using rabbit erythrocytes, as previously described (Bernheimer 1988). LAC was cultured for 5 h at 37°C in 100 μl TSB with PLNA34 (at 3·125, 6·25 and 12·5 μmol l^{-1}) or PBS. Bacteria were removed by centrifugation and culture supernatants were filtered, aliquoted and stored at −80°C. Culture filtrates were diluted 1 : 1 with PBS and were added to washed rabbit erythrocytes (4% suspension in 0·01 mol l^{-1} PBS (pH 7·2)). Samples were subsequently incubated for 30 min at 37°C, followed by centrifugation at 5500 × ***g*** for 1 min. Triton X-100 (1%) was used as a positive control, while PBS was used as a negative control. The absorbance of the supernatants was determined at 545 nm. The percentage of haemolysis was calculated as % haemolysis = (OD_{545}(sample)−average OD_{545}(negative control))/(average OD_{545}(positive control)−average OD_{545}(negative control)) × 100%. Assays were performed in triplicate.

Neutrophil preparation and lysis assay

Human neutrophils were isolated from the blood of healthy volunteers by standard Ficoll/Histopaque gradient centrifugation. PMN lysis by LAC culture supernatants was determined as previously described (Wang *et al.* 2007). Briefly, culture filtrate (50% dilution) was added

Table 1 Primers used in RT-qPCR

Primer	Sequence (5′–3′)	Slope*	PCR efficiency (%)†
16S rRNA-F	GCCGTAAACGATGAGTGCTAA	−3·282	101·7
16S rRNA-R	CGAATTAAACCACATGCTCCA		
RNAIII-F	AATACATAGCACTGAGTCCAAGG	−3·194	105·6
RNAIII-R	TGGATTATCGACACAGTGAACA		
agrA-F	TGATAATCCTTATGAGGTGCTT	−3·381	97·6
agrA-R	CACTGTGACTCGTAACGAAAA		
hla-F	CTGAAGGCCAGGCTAAACCACTTT	−3·568	90·7
hla-R	GAACGAAAGGTACCATTGCTGGTCA		
psmα-F	TATCAAAAGCTTAATCGAACAATTC	−3·462	94·5
psmα-R	CCCCTTCAAATAAGATGTTCATATC		
psmβ-F	CTAGCAGAAGCAATCGCAAA	−3·148	107·8
psmβ-R	AACCCACACCGTTAGCAACG		
pvl-F	CCAATAAATTCTGGATTGAAGTTACCT	−3·345	99
pvl-R	GCTCAAGACAAAGCAACTTAAATGC		

*The slope was from the calibration curve, where twofold dilutions of cDNA from the PBS control group were analysed by qPCR for each gene.
†PCR efficiency = $(10^{-1/\text{slope}} - 1) \times 100\%$.

to the wells of a 96-well microtitre plate containing 10^6 PMNs and plates were incubated at 37°C. Neutrophils with 1% Triton X-100 were used as a positive control; neutrophils incubated with RPMI 1640 (with 5 mmol l^{-1} HEPES) were used as a negative control. After 2 h, PMN lysis was determined by the release of lactate dehydrogenase using the Cytotoxicity Detection Kit^{PLUS} (Roche Diagnostics Ltd., Shanghai, China).

Chemotactic assay

The chemotaxis of neutrophils towards staphylococcal culture filtrates was determined using fluorescently labelled neutrophils that migrated through a 3-μm-pore-size polycarbonate transwell filter as previously described (de Haas *et al.* 2004). In brief, 5×10^6 neutrophils per ml were labelled with 3·3 μmol l^{-1} 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluoresceinacetoxymethyl ester (BCECF-AM) (Molecular Probes, Inc., Eugene, OR, USA) for 30 min at room temperature. Cells were washed and resuspended in HBSS/0·05% BSA, following which 5×10^5 neutrophils were added to the upper compartment of the Transwell system. The upper compartment containing the neutrophils was subsequently placed into a well containing 600 ml of buffer or culture supernatants (1% dilution). After incubation at 37°C for 1 h in a 5% CO_2 atmosphere, inserts were removed and fluorescence intensity of the wells was analysed using a FL600 fluorescence reader (Bio Tek Instruments, Winooski, VT, USA), with excitation at 485 nm and emission at 530 nm. Control wells of BCECF-labelled cells were included in order to set a maximum fluorescence value (100%). The fluorescence values of each sample were expressed relative to the total and migration towards the control buffer was subtracted, to give the percentage of cells that actively migrated across the membrane.

Mouse skin infection model

Male C57BL/6J mice at 6–8 weeks of age and weighing 18–22 g were used in this study. All experimental and animal care procedures were approved by the Animal Care and Use Committee of the Fourth Military Medical University. All methods were carried out strictly according to the approved guidelines.

Before each experiment, animals were shaved around the injection site. The mouse subcutaneous abscess model was performed as described previously (Bunce *et al.* 1992). Briefly, *S. aureus* were cultured to mid-exponential phase (~3 h), washed once with PBS, then injected into 10 mice per group at 1×10^7 CFU *S. aureus* in a final volume 50 μl PBS that had been mixed with cytodex beads (Sigma). For *agr* inhibition studies, PLNA34 (40 μmol l^{-1}) or scrambled PLNA34 (40 μmol l^{-1}) was mixed with the bacteria immediately before subcutaneous injection into mice. After infection, five mice in each group were examined daily for skin lesions for a total of 14 days. Skin lesion dimensions were measured at each inspection with a caliper. The area of each lesion was calculated by multiplying the length (L) of each lesion by the measured width (W) value. On the fourth day after infection, the abscesses from the other five mice were excised, and part of the excised tissue was fixed in 10% formalin (Sigma). Paraffin embedding and haematoxylin and eosin staining were performed as previously described. CFU per abscess were determined by homogenizing excised lesions in PBS plus 0·5% Triton X-100 and plating serial dilutions.

Statistical analyses

Statistical analyses were performed with GRAPH PAD PRISM 5 software (GraphPad Software, Inc., La Jolla, CA, USA) using one-way analysis of variance with Bonferroni or Dunnett post-tests, as appropriate.

Results

Design and synthesis of antisense oligonucleotides targeting *agrA* mRNA

BLASTN results indicated that the *agrA* coding region was very highly conserved among *S. aureus* strains (Table 2), which suggested that *agrA* would be a specific antisense target. Based on Primer Premier 5.0 and RNA structure 4.5 analyses, two potential antisense oligonucleotides were selected and their target regions and binding parameters are shown in Fig. 1 and Table 3. These two antisense

Table 2 Homology of *agrA* gene coding region among *Staphylococcus* sp.

Organism	Genbank	Identity (%)
Staphylococcus aureus ssp. *aureus*		
M013	CP003166·1	99
LGA251	FR821779·1	99
JKD6159	CP002114·2	99
ED133	CP001996·1	99
TW20	FN433596·1	99
USA300 TCH1516	CP000730·1	99
USA300 FPR3757	CP000255·1	99
Mu3	AP009324·1	99
Str. Newman	AP009351·1	99
MSSA476	BX571857·1	99
MW2	BA000033·2	99
Mu50	BA000017·4	98
MRSA252	BX571856·1	98

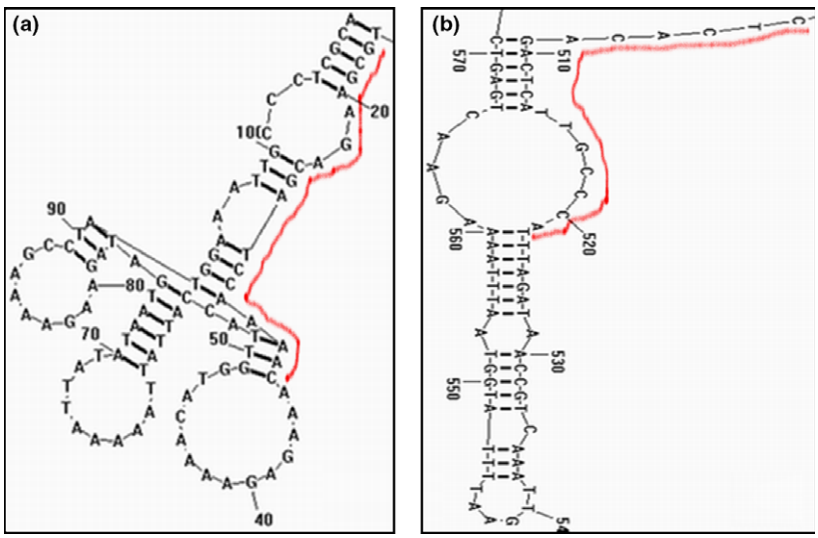

Figure 1 The target region of (a) PLNA34 (nucleotides 17–34) and (b) PLNA522 (nucleotides 503–522).[Colour figure can be viewed at wileyonlinelibrary.com]

Table 3 Binding parameters predicted by RNA structure 4.6 and Primer Premier 5.0

			Parameters†					
Antisense (AS)	Target site*	Length (nt)	Overall ΔG	Duplex ΔG	Oligo-self ΔG	Oligo-Oligo ΔG	GC (%)	*Tm* (°C)
AS34	17–34	18	−13	−22·3	−0·4	−4·7	50	70·2
AS522	503–522	20	−13·3	−23·1	0	−3·7	50	68·7

*Numbering from the first base of *agrA* gene (5′–3′).
†ΔG (kcal per mol) means free energy.

oligonucleotides were synthesized as LNAs and covalently conjugated with cell penetrating peptide-$(KFF)_3K$, resulting in PLNA34 and PLNA522 (Table 4).

Influence of PLNAs on bacterial growth

As shown in Fig. 2, the growth curves for LAC strain cultured with 12·5–40 μmol l^{-1} of either PLNA34 or PLNA522 were the same as the PBS control group, indicating that neither PLNA34 nor PLNA522 had an antibacterial effect on LAC *in vitro*. The scrambled PLNA34 used in the following experiment also did not exhibit any antibacterial activity (data not shown). In addition, MIC values further confirmed that PLNA34 and PLNA522 also did not have bacteriostatic activity at concentrations <50 μmol l^{-1} (Table S1).

Effect of PLNAs on disrupting *agr* QS

To evaluate *agr* QS inhibition caused by PLNAs, RT-qPCR was performed to quantify transcriptional of the target gene *agrA* and the effector molecule *RNAIII*. Both PLNA34 and PLNA522 decreased the *agrA* transcription at a concentration of <12·5 μmol l^{-1}, while PLNA34 had a much stronger inhibitory effect than PLNA522 (Fig. 3a).

Table 4 Name and sequence of CPP-LNAs

Name	Sequence
PLNA34	KFFKFFKFFK-Cys-SMCC-C6-5′G+*T+T*TTG+GA*TC+GT+CT*T+C+G+*C
PLNA522	KFFKFFKFFK-Cys-SMCC-C6-5′A+*T*GGG*CAA*TG+AG+TC*TGT*G+A+*G
Scrambled PLNA34	KFFKFFKFFK-Cys-SMCC-C6-5′T+*G+*CAT+TTC*TGC+TGTG+T+*C*G

A cysteine is used in the conjugate sequence.
+Represents LNA base, *represents phosphorothioate linkage.

Furthermore, similar results were observed for the effector molecule of *agr*, *RNAIII* (Fig. 3b). These results indicated that PLNA34 had a greater inhibitory effect on *agr* QS than PLNA522. Moreover, the inhibition caused by PLNA34 was concentration-dependent (Fig. S2). As such, PLNA34 was chosen for further testing.

Effect of PLNA34 on toxin gene expression

To further investigate the downstream effects of *agr* inhibition, we measured the mRNA expression levels of those toxins whose expression is upregulated by *agr*. As expected, expression of *psmα* and *psmβ*, which are AgrA-dependent

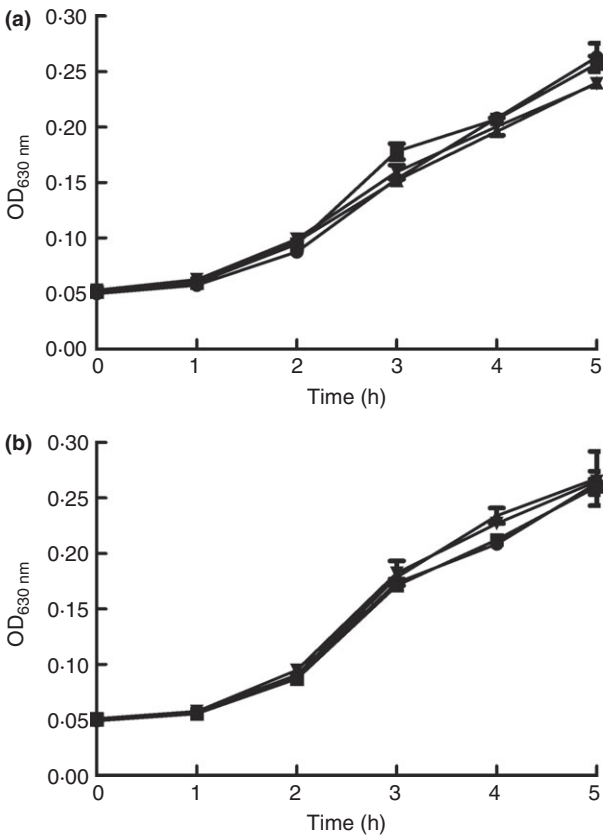

Figure 2 Growth of LAC (USA300) in tryptone soy broth with anti-*agrA* PLNAs. (a) Anti-*agrA* PLNA34 or (b) anti-*agrA* PLNA522 was added to LAC cell cultures to a final concentration of (–■–) 12·5, (–▲–) 25, or (–▼–) 40 μmol^{-1}. Bacterial cultures were treated with (–●–) PBS as a control. The growth was measured spectrophotometrically at 630 nm. The data are shown as means ± SEM (n = 3).

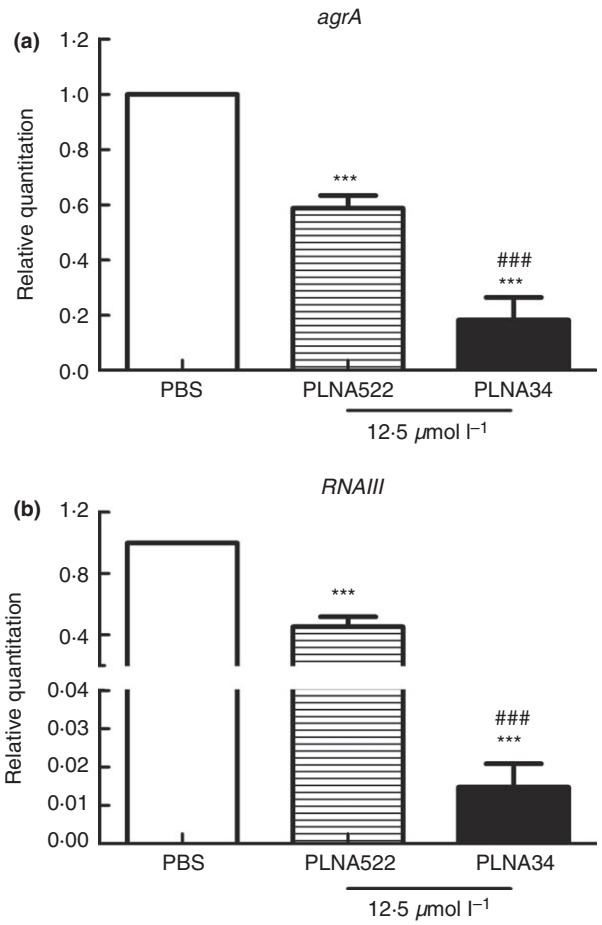

Figure 3 The inhibition of (a) *agrA* and (b) *RNAIII* transcription by PLNA34 and PLNA522. Triplicate bacterial cultures were grown for 5 h in the presence of PLNA34 or PLNA522 at a concentration of 12·5 μmol l^{-1}. Additional cell cultures were treated with PBS as a control. After 5 h of growth, total RNA was extracted from the treated bacteria. Transcription levels of *agrA* and *RNAIII* were detected by real-time PCR. The data are shown as means ± SEM (n = 3). ***P < 0·001 *vs* PBS control. ###P < 0·001 *vs* PLNA522-treated group.

toxin genes, was decreased in a concentration-dependent manner (Fig. 4a,b). Furthermore, *RNAIII*-dependent toxin gene expression, including that of *hla* and *pvl*, was also decreased (Fig. 4c,d). In contrast, scrambled PLNA34 had no inhibition on the expression of these genes. The amount of α-toxin, the translational product of *hla*, in culture supernatants was also drastically decreased by PLNA34 (Fig. S3). These results demonstrated that PLNA34-dependent knock-down of *agrA* suppressed the expression of *agr*-regulated toxin genes.

Activity of PLNA34-treated LAC culture filtrates on erythrocytes and neutrophils

We then determined the haemolytic effect of LAC culture filtrates on rabbit erythrocytes, and found that culture filtrates from PLNA34-treated cells had decreased haemolytic activity (Fig. 5a), which is consistent with the Western blotting result (Fig. S3). Compared to the PBS control or the scrambled group, culture filtrates of PLNA34-treated LAC showed significantly reduced lysis of human neutrophils in a concentration-dependent manner (Fig. 5b). Furthermore, PLNA34-treated culture supernatants were shown to have a reduced capacity to induce chemotaxis in human neutrophils in a concentration-dependent manner (Fig. 5c).

Anti-*agrA* PLNA34 reduces LAC-induced skin injury in mice

As PLNA34 was shown to decrease *agrA* mRNA levels and disrupt *agr* QS, we wanted to investigate whether PLNA34 had a protective effect on *S. aureus* infections *in vivo*. Subcutaneous injection of LAC resulted in the formation of large abscesses, which were significantly smaller in size

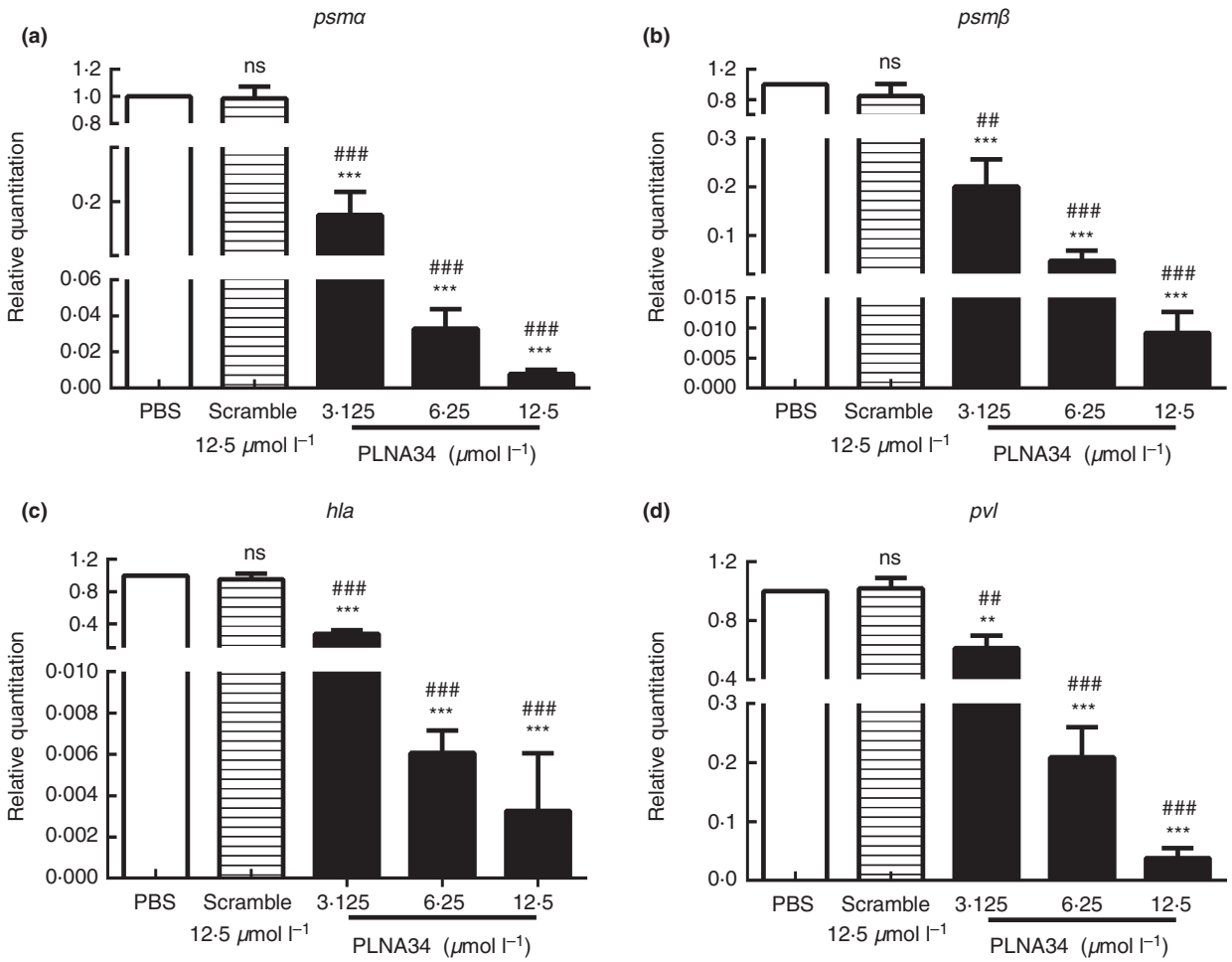

Figure 4 Dose-dependent inhibition of *agr*-regulated gene transcription by PLNA34. As in Fig. 2, triplicate LAC cell cultures were grown for 5 h in the presence of PLNA34 at different concentrations (final concentration 3·125, 6·25 and 12·5 μmol l^{-1}) or scrambled PLNA34 (final concentration 12·5 μmol l^{-1}). After 5 h of growth, total RNA was extracted from the treated bacteria. Transcription levels of (a) *psmα*, (b) *psmβ*, (c) *hla* and (d) *pvl* were detected by RT-qPCR. The data are shown as mean ± SEM (n = 3). $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ *vs* PBS control, $^{\#\#}P < 0{\cdot}01$, $^{\#\#\#}P < 0{\cdot}001$ *vs* scrambled control.

upon the addition of 40 μmol l^{-1} PLNA34 at the time of injection (Fig. 6a). Animals infected with LAC developed necrosis of the epidermis and dermis with epidermal ulceration, which was not seen in mice that received LAC in combination with PLNA34 (Fig. 6c). Furthermore, the protective effects were associated with a >4 log unit decrease in CFU at the infection site (Fig. 6b).

Discussion

CA-MRSA has become a significant problem and is associated with debilitating skin and soft tissue infections (SSTIs), due to its hypervirulence and invasive ability. Toxins are important weapons secreted by CA-MRSA and contribute to its pathogenesis. α-Toxin, encoded by *hla*, causes lysis of many cell types, such as erythrocytes and macrophages but not neutrophils, and is implicated in breaching of the epithelial barrier. α-Toxin also has pro-inflammatory activity, including induction of the inflammasome and generation of highly pro-inflammatory cytokines such as IL-1β and IL-18 (Craven *et al.* 2009). The PSMs are a group of peptide toxins, of which PSMα has strong cytolytic activity towards neutrophils and many other cell types in the micromolar range (Peschel and Otto 2013). At nanomolar concentrations, PSMα stimulates neutrophils and initiates pro-inflammatory responses through human formyl peptide receptor 2 (FPR2/ALX), which includes neutrophil chemoattraction and activation, and the release of IL-8 (Peschel and Otto 2013). In addition, leukocidins, including Panton–Valentine leukocidin and LukAB, also have a strong cytolytic activity against a variety of host immune cells.

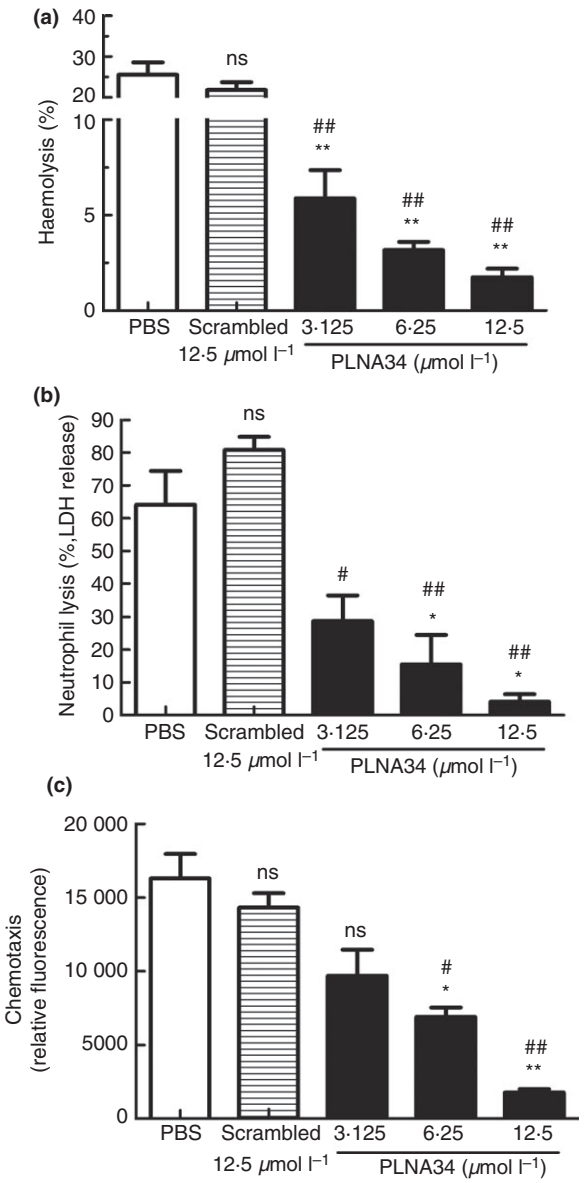

Figure 5 Anti-*agrA* PLNA34 inhibits secreted virulence factor production. As in Fig. 3, after 5 h of growth with different concentrations of PLNA34, LAC culture supernatant was collected. (a) Haemolytic activity of LAC culture filtrates was determined by incubation with rabbit erythrocytes. PBS was used as a negative control and 1% TritonX-100 was used as a positive control. (b) Neutrophil lysis by LAC culture filtrates was determined by lactate dehydrogenase release assay. (c) Neutrophil chemotaxis after the addition of LAC culture filtrates was measure by determining the number of fluorescently labelled neutrophils that migrated through a transwell filter. Culture filtrates from PBS-treated group were used as a control. The data are shown as mean ± SEM ($n = 3$). $^{***}P < 0{\cdot}001$, $^{**}P < 0{\cdot}01$, $^{*}P < 0{\cdot}05$ *vs* PBS control, $^{\#\#}P < 0{\cdot}01$, $^{\#}P < 0{\cdot}05$ *vs* scrambled control.

The *agr* QS is a key regulator that broadly controls expression of many virulence genes in *S. aureus* in a *RNAIII*-dependent or AgrA-dependent manner (Queck *et al.* 2008), including those toxins mentioned above. AgrA is an essential transcription factor for *agr*, as well as for its downstream effector molecule *RNAIII*. In this study, we firstly employed antisense oligonucleotide analogues, LNA, targeting *agrA* to disrupt *agr* QS in *S. aureus*. LNA, as a third generation oligonucleotide, has many favourable properties, including resistance to nucleases and high affinity to target RNAs. Our previous study showed that cell penetrating peptides-$(KFF)_3K$ can facilitate the introduction of LNA into *S. aureus* cells (Meng *et al.* 2015). Accordingly, we designed and synthesized PLNA by conjugating LNA with $(KFF)_3K$.

Our results showed that PLNA34 specifically and significantly reduced *agrA* mRNA levels with no bacteriostatic or bactericidal activity. Also, *agr* inactivity was demonstrated by dramatically decreased *RNAIII* expression. As an expected follow-on effect of *agr* inhibition, reduced expression of toxin genes upregulated by *RNAIII*, including *hla* and *pvl*, was observed after PLNA34 treatment, as well as *psmα* and *psmβ*, which are directly controlled by AgrA. Furthermore, the culture supernatants of PLNA34-treated LAC had significantly decreased cytolytic activity towards both rabbit erythrocytes and human neutrophils, and exhibited decreased neutrophil chemoattraction.

In the mouse subcutaneous infection model, co-injection of LAC with PLNA34 led to a significant decrease in the size of lesions, with a reduction in bacterial load at infection site and dramatically decreased neutrophil infiltration, implying the potential of PLNA34 to attenuate CA-MRSA pathogenesis by targeting *agrA in vivo*. There are two potential mechanisms for the protective effect of PLNA34. On the one hand, the destruction of immune cells, especially neutrophils, caused by cytolytic toxins is inhibited by PLNA34, which allows the bacteria to be more efficiently eliminated. On the other hand PLNA34 suppressed the production of pro-inflammatory virulence factors, especially PSMs, which would at least partially account for the decreased neutrophil influx and decreased tissue damage caused by excessive inflammation.

Although developing *agr* QS inhibitors is an alternative strategy to control MRSA infections, it has its own limitations. Indeed *agr* plays a dominant role in acute infection, especially SSTIs (Wright *et al.* 2005). However, enhanced biofilm formation and persistent bacteraemia have also been observed in *agr* defective strains (Vuong *et al.* 2000; Fowler *et al.* 2004). In general, *agr* can upregulate many virulence determinants, such as toxins and exoenzymes, but can also downregulate surface proteins, such as adhesins (Le and Otto 2015). However, the first step in biofilm formation is colonization, which requires surface proteins, while degradative exoenzymes facilitate biofilm detachment. That means *agr* mutants could easily establish a biofilm, but the cells may resist detachment. Therefore,

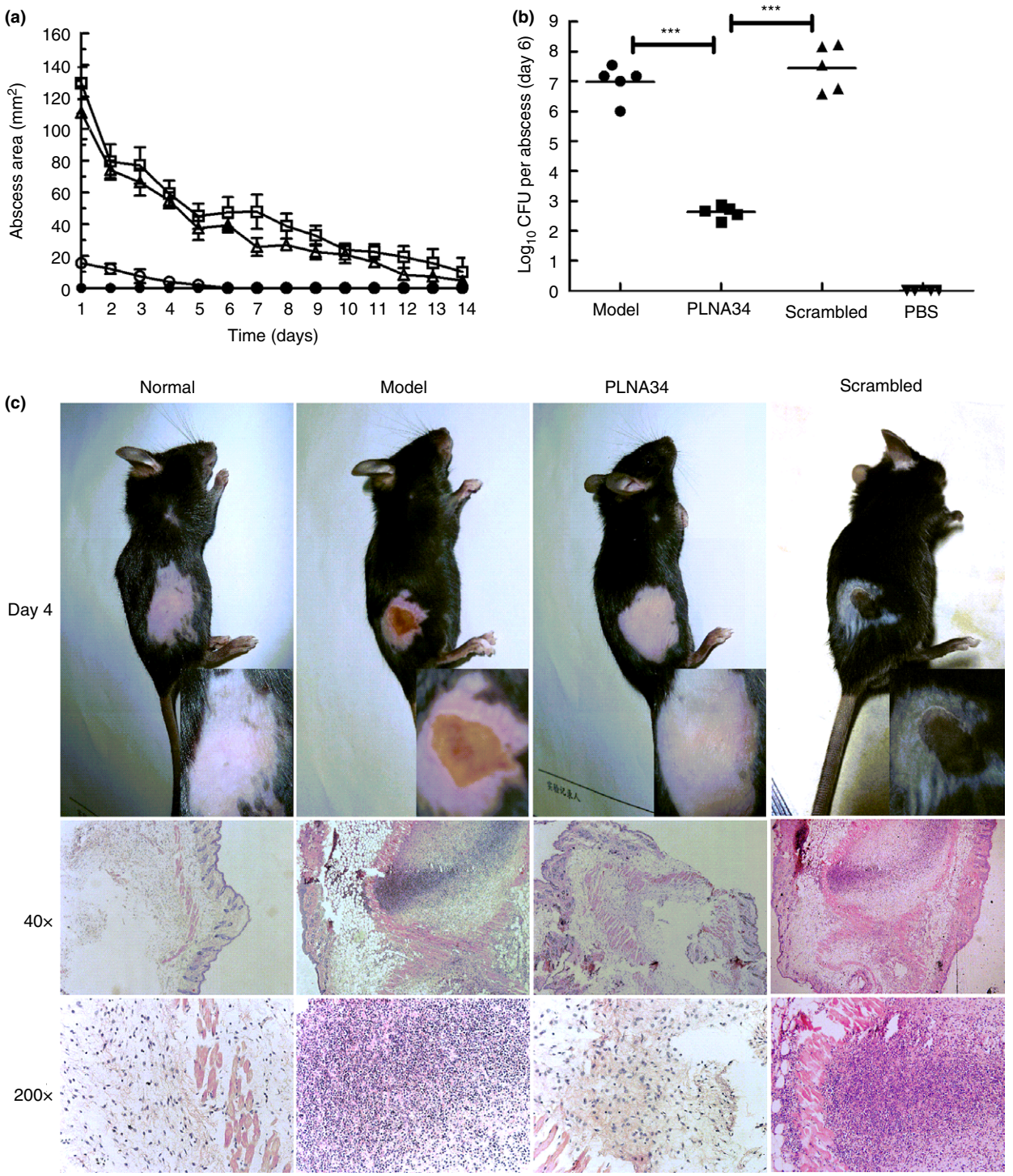

Figure 6 Protective effect of PLNA34 on *Stapylococcus aureus* mouse skin infection. Subcutaneous injections were made into the right flank of C57 BL/6J mice (n = 10 per group). Mice were inoculated with 10^7 CFU of LAC, 10^7 CFU of LAC + 40 μmol l^{-1} PLNA34, 10^7 CFU of LAC+40 μmol l^{-1} scrambled PLNA34 or PBS. (a) Skin lesion areas were measured daily with calipers (n = 5). (—●—) PBS; (—□—) model; (—△—) scrambled; (—○—) PLNA34. (b) Bacterial burden in the lesion was assessed by colony enumeration (n = 5). (c) Gross pathology and histopathological evaluation of representative lesions in mice 4 days after infection. The data are shown as mean ± SEM. ***P < 0·001 *vs* model or scrambled control.[Colour figure can be viewed at wiley onlinelibrary.com]

infection may occur more readily once *agr* mutant bacteria are released from a biofilm by mechanical measures, such as flushing in the bloodstream. Thus, instead of treatment, inhibitors of *agr* signalling may be beneficial in biofilm-related or chronic diseases. Recently, the down-regulation by *agr* of toxicity has been shown to be important in the development of bacteraemia, due to the increased fitness of low toxin producing strains in human serum (Dunn *et al.* 2015). Loss of *agr* function has been linked with reduced vancomycin susceptibility and low-level resistance to platelet microbicidal protein (Sakoulas *et al.* 2005). Accordingly, *agr* appears to have divergent roles in different types of infection. Nevertheless, *agr* inhibitors may be useful as drugs for specifically decreasing acute toxicity in some *S. aureus* infections (Le and Otto 2015).

So far, most *agr* inhibitors were assessed by co-injection with bacteria in a mouse skin infection model. Compared to co-injection, delayed administration of savarin only reduced the severity of dermonecrosis and promoted bacterial clearance, but did not reduce total abscess size (Sully *et al.* 2014). It seems that the early phase of infection is important for the bacteria. Therefore, we propose that *agr* inhibitors may provide the best protection if topically administrated as early as possible once infection occurs or even before infection. As *agr* inhibitors are not bacteriostatic or bactericidal, improved protective effects may be observed when *agr* inhibitors and some antibiotics are administered together. Recently, antibody-antibiotic conjugates have been developed to kill intracellular *S. aureus* (Lehar *et al.* 2015), which provides a new concept to combat bacterial infections. Accordingly, another promising avenue may be to conjugate *agr* inhibitors, like our PLNAs or other molecules, to antibiotics to form novel anti-MRSA agents that could decrease virulence, as well as inhibit bacteria.

In summary, the results presented in this study show that *agrA* is a promising antisense target for the development of *agr* QS inhibitors to combat MRSA infections, in particular SSTIs or other acute infections. The inhibition of *agrA* expression by PLNA34 not only attenuates the pathogenicity of MRSA, but also allows the host immune system to eliminate the invading bacteria.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81273555). We thank Dr Michael Otto (Laboratory of Human Bacterial Pathogenesis, NIAID, NIH, USA) for providing *S. aureus* USA300 LAC strain.

Conflict of Interest

The authors declare no competing financial interest.

References

Bernheimer, A.W. (1988) Assay of hemolytic toxins. *Methods Enzymol* **165**, 213–217.

Bunce, C., Wheeler, L., Reed, G., Musser, J. and Barg, N. (1992) Murine model of cutaneous infection with gram-positive cocci. *Infect Immun* **60**, 2636–2640.

CDC. (2013) Antibiotic resistance threats in the United States.

Chambers, H.F. and Deleo, F.R. (2009) Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat Rev Microbiol* **7**, 629–641.

Cheung, G.Y., Wang, R., Khan, B.A., Sturdevant, D.E. and Otto, M. (2011) Role of the accessory gene regulator agr in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis. *Infect Immun* **79**, 1927–1935.

Craven, R.R., Gao, X., Allen, I.C., Gris, D., Bubeck Wardenburg, J., McElvania-Tekippe, E., Ting, J.P. and Duncan, J.A. (2009) *Staphylococcus aureus* alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS ONE* **4**, e7446.

Diekema, D.J., Pfaller, M.A., Schmitz, F.J., Smayevsky, J., Bell, J., Jones, R.N. and Beach, M. (2001) Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. *Clin Infect Dis* **32**(Suppl 2), S114–S132.

Dunn, J.C., Halenar, L.B., Davies, T.G., Cristobal-Azkarate, J., Reby, D., Sykes, D., Dengg, S., Fitch, W.T. *et al.* (2015) Evolutionary trade-off between vocal tract and testes dimensions in Howler monkeys. *Curr Biol* **25**, 2839–2844.

Fowler, V.G., Sakoulas, G., McIntyre, L.M., Meka, V.G., Arbeit, R.D., Cabell, C.H., Stryjewski, M.E., Eliopoulos, G.M. *et al.* (2004) Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with agr dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidal protein. *J Infect Dis* **190**, 1140–1149.

de Haas, C.J., Veldkamp, K.E., Peschel, A., Weerkamp, F., Van Wamel, W.J., Heezius, E.C., Poppelier, M.J., Van Kessel, K.P. *et al.* (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J Exp Med* **199**, 687–695.

Khan, B.A., Yeh, A.J., Cheung, G.Y. and Otto, M. (2015) Investigational therapies targeting quorum-sensing for the treatment of *Staphylococcus aureus* infections. *Expert Opin Investig Drugs* **24**, 689–704.

Kobayashi, S.D., Malachowa, N., Whitney, A.R., Braughton, K.R., Gardner, D.J., Long, D., Bubeck Wardenburg, J., Schneewind, O. *et al.* (2011) Comparative analysis of USA300 virulence determinants in a rabbit model of skin and soft tissue infection. *J Infect Dis* **204**, 937–941.

Le, K.Y. and Otto, M. (2015) Quorum-sensing regulation in staphylococci—an overview. *Front Microbiol* **6**, 1174.

Lehar, S.M., Pillow, T., Xu, M., Staben, L., Kajihara, K.K., Vandlen, R., DePalatis, L., Raab, H. *et al.* (2015) Novel antibody-antibiotic conjugate eliminates intracellular *S. aureus*. *Nature* **527**, 323–328.

Matveeva, O.V., Mathews, D.H., Tsodikov, A.D., Shabalina, S.A., Gesteland, R.F., Atkins, J.F. and Freier, S.M. (2003) Thermodynamic criteria for high hit rate antisense oligonucleotide design. *Nucleic Acids Res* **31**, 4989–4994.

Meng, J., Da, F., Ma, X., Wang, N., Wang, Y., Zhang, H., Li, M., Zhou, Y. *et al.* (2015) Antisense growth inhibition of methicillin-resistant *Staphylococcus aureus* by locked nucleic acid conjugated with cell-penetrating peptide as a novel FtsZ inhibitor. *Antimicrob Agents Chemother* **59**, 914–922.

Moellering, R.C. Jr (2012) MRSA: the first half century. *J Antimicrob Chemother* **67**, 4–11.

Otto, M. (2013) Community-associated MRSA: what makes them special? *Int J Med Microbiol* **303**, 324–330.

Park, J., Jagasia, R., Kaufmann, G.F., Mathison, J.C., Ruiz, D.I., Moss, J.A., Meijler, M.M., Ulevitch, R.J. *et al.* (2007) Infection control by antibody disruption of bacterial quorum sensing signaling. *Chem Biol* **14**, 1119–1127.

Peschel, A. and Otto, M. (2013) Phenol-soluble modulins and staphylococcal infection. *Nat Rev Microbiol* **11**, 667–673.

Queck, S.Y., Jameson-Lee, M., Villaruz, A.E., Bach, T.H., Khan, B.A., Sturdevant, D.E., Ricklefs, S.M., Li, M. *et al.* (2008) RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. *Mol Cell* **32**, 150–158.

Reuter, K., Steinbach, A. and Helms, V. (2016) Interfering with bacterial quorum sensing. *Perspect Medicin Chem* **8**, 1–15.

Sakoulas, G., Eliopoulos, G.M., Fowler, V.G. Jr, Moellering, R.C. Jr, Novick, R.P., Lucindo, N., Yeaman, M.R. and Bayer, A.S. (2005) Reduced susceptibility of *Staphylococcus aureus* to vancomycin and platelet microbicidal protein correlates with defective autolysis and loss of accessory gene regulator (agr) function. *Antimicrob Agents Chemother* **49**, 2687–2692.

Sully, E.K., Malachowa, N., Elmore, B.O., Alexander, S.M., Femling, J.K., Gray, B.M., DeLeo, F.R., Otto, M. *et al.* (2014) Selective chemical inhibition of agr quorum sensing in *Staphylococcus aureus* promotes host defense with minimal impact on resistance. *PLoS Pathog* **10**, e1004174.

Tal-Gan, Y., Stacy, D.M., Foegen, M.K., Koenig, D.W. and Blackwell, H.E. (2013) Highly potent inhibitors of quorum sensing in *Staphylococcus aureus* revealed through a systematic synthetic study of the group-III autoinducing peptide. *J Am Chem Soc* **135**, 7869–7882.

Vuong, C., Saenz, H.L., Gotz, F. and Otto, M. (2000) Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J Infect Dis* **182**, 1688–1693.

Wang, R., Braughton, K.R., Kretschmer, D., Bach, T.H., Queck, S.Y., Li, M., Kennedy, A.D., Dorward, D.W. *et al.* (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* **13**, 1510–1514.

Winer, J., Jung, C.K., Shackel, I. and Williams, P.M. (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem* **270**, 41–49.

Wright, J.S. 3rd, Jin, R. and Novick, R.P. (2005) Transient interference with staphylococcal quorum sensing blocks abscess formation. *Proc Natl Acad Sci USA* **102**, 1691–1696.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Expression levels of *16S rRNA* in different treatment groups. Gene expression levels are presented as absolute Cq values. The concentration of PLNA522 and the scrambled PLNA34 was 12·5 μmol l^{-1}.

Figure S2 Decreased expression of (a) *agrA* and (b) *RNAIII* by PLNA34 in a concentration-dependent manner.

Figure S3 α-toxin present in the LAC culture supernatant was detected by Western blotting.

Table S1 MICs of anti-agrA PLNAs against LAC in Mueller-Hinton broth medium.