J Antimicrob Chemother 2015; **70**: 1443–1452
doi:10.1093/jac/dku547 Advance Access publication 5 January 2015

Journal of Antimicrobial Chemotherapy

Early *agr* activation correlates with vancomycin treatment failure in multi-clonotype MRSA endovascular infections

Wessam Abdelhady[1]†, Liang Chen[2]†, Arnold S. Bayer[1,3], Kati Seidl[4], Michael R. Yeaman[1,3], Barry N. Kreiswirth[2] and Yan Q. Xiong[1,3]*

[1]Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA, USA; [2]Public Health Research Institute, NJMS-Rutgers University, Newark, NJ, USA; [3]David Geffen School of Medicine at UCLA, Los Angeles, CA, USA; [4]University Hospital Zurich, University of Zurich, Zurich, Switzerland

*Corresponding author. Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, 1124 West Carson Street, Bldg RB-2, Room 231, Torrance, CA 90502, USA. Tel: +1-310-222-3545; Fax: +1-310-782-2016; E-mail: yxiong@ucla.edu
†Contributed equally to this study.

Received 24 September 2014; returned 3 November 2014; revised 19 November 2014; accepted 3 December 2014

Objectives: Persistent MRSA infections are especially relevant to endovascular infections and correlate with suboptimal outcomes. However, the virulence signatures of *Staphylococcus aureus* that drive such persistence outcomes are not well defined. In the current study, we investigated correlations between accessory gene regulator (*agr*) activation and the outcome of vancomycin treatment in an experimental model of infective endocarditis (IE) due to MRSA strains with different *agr* and clonal complex (CC) types.

Methods: Twelve isolates with the four most common MRSA CC and *agr* types (CC5-*agr* II, CC8-*agr* I, CC30-*agr* III and CC45-*agr* I) were evaluated for heterogeneous vancomycin-intermediate *S. aureus* (hVISA), *agr* function, *agrA* and *RNAIII* transcription, *agr* locus sequences, virulence and response to vancomycin in the IE model.

Results: Early *agr RNAIII* activation (beginning at 2 h of growth) in parallel with strong δ-haemolysin production correlated with persistent outcomes in the IE model following vancomycin therapy. Importantly, such treatment failures occurred across the range of CC/*agr* types studied. In addition, these MRSA strains: (i) were vancomycin susceptible *in vitro*; (ii) were not hVISA or vancomycin tolerant; and (iii) did not evolve hVISA phenotypes or perturbed δ-haemolysin activity *in vivo* following vancomycin therapy. Moreover, *agr* locus sequence analyses revealed no common point mutations that correlated with either temporal *RNAIII* transcription or vancomycin treatment outcomes, encompassing different CC and *agr* types.

Conclusions: These data suggest that temporal *agr RNAIII* activation and *agr* functional profiles may be useful biomarkers to predict the *in vivo* persistence of endovascular MRSA infections despite vancomycin therapy.

Keywords: bacteria, antibiotics, resistance

Introduction

Staphylococcus aureus is the most common cause of endovascular infections, including infective endocarditis (IE), and the second most frequent cause of bacteraemia.[1,2] Invasive infections such as IE, particularly those caused by MRSA strains, are associated with a high mortality (15%–40%).[3–5] In addition, the high rates of clinical failure on vancomycin (even with CLSI-defined 'susceptible' strains) and the growing problem of daptomycin non-susceptibility, have further complicated the management of patients with invasive MRSA infections.[5,6]

The pathogenesis of *S. aureus* is largely controlled by regulatory networks composed of: (i) global regulons (e.g. *sarA*); (ii) two-component regulatory systems (e.g. *agr* and *saeRS*); and (iii) downstream effector genes (e.g. *hla*).[7,8] Central to the orchestration of this regulatory network is the quorum-sensing operon *agr*. Of note, *agr* consists of two transcription domains, RNAII and RNAIII, driven by the divergent promoters P2 and P3, respectively.[9] RNAII encodes a quorum-sensing system and RNAIII is the effector molecule of the *agr* system. *In vitro*, *agr* down-regulates many pivotal cell surface proteins involved in tissue colonization while up-regulating exoproteins associated with host cell damage.[9] *In vitro* studies have suggested that the temporal activation of *agr* is an important determinant of *agr* functionality; i.e. earlier versus later *agr* activation correlates with intact versus attenuated function, respectively.[6,10] As most of

the available data on *agr* expression and regulatory impacts have only been obtained *in vitro*, an important question relates to how such temporal regulatory functions translate to the *in vivo* scenario, particularly in the context of endovascular MRSA infections that persist versus resolve after antibiotic treatment.

We previously demonstrated a positive correlation between early-onset *agr RNAIII* activation and resistance to vancomycin treatment in an experimental model of IE.[6] Although intriguing, these initial studies were somewhat limited: (i) they were restricted to strains from only two clonotypes (CC45-*agr* I and CC5-*agr* II); and (ii) they did not account for other genotypic influences on outcomes such as variations of *agr* types. Thus, it remains unknown whether the relationship between the temporal *RNAIII* activation profiles and antibiotic treatment outcomes is CC and/or *agr* type-specific in endovascular infection. Therefore, in the current investigation, we sought to validate and extend upon our prior observations, using a broad range of the most common clonotypes causing invasive MRSA infections such as IE.[11–13] The overarching goals of this project were: (i) to assess key genetic strategies used by MRSA to resist antimicrobial therapy (e.g. early *agr* activation); and (ii) to identify novel biomarkers that correlate with the phenotype of persistent endovascular MRSA infection.

Materials and methods

Bacterial strains and growth conditions

In the current study, we selected two CC5-*agr* II, four CC8-*agr* I and two CC30-*agr* III MRSA clinical isolates; in addition, we chose four previously sequenced reference strains of *S. aureus* (one for each CC type, including CC45) as relevant genetic controls (B. N. Kreiswirth, unpublished data; Table 1). These strains were from a multinational clinical trial collection and other sources[14] and were pre-selected based on their relative δ-haemolysin activities as outlined below. Unless otherwise stated, all *S. aureus* strains were grown at 37°C either in tryptic soy broth (TSB; Difco) or TSB agar plates. Stocks were kept at −80°C in brain heart infusion (Difco) broth supplemented with 10% glycerol.

agr, SCCmec, spa, CC typing and agr locus sequence

The *agr* group was determined by a multiplex PCR assay described elsewhere,[15] while the *SCCmec* type was examined using a multiplex real-time PCR published previously.[16] The *spa* typing was conducted according to a protocol described before; the corresponding clonal complex (CC) of each strain was inferred based on the *spa* types.[17] Full-length *agr* locus sequencing was performed as previously described.[6]

MICs, kill curves and population analyses

The determination of vancomycin MICs was conducted by broth microdilution as recommended by the CLSI.[18] *In vitro*, vancomycin kill curves and population analyses were carried out to detect vancomycin tolerance and heterogeneous vancomycin-intermediate *S. aureus* (hVISA), respectively.[19] All *in vitro* experiments were performed at least twice for each strain on different days.

δ-Haemolysin activity

δ-Haemolysin activity was assayed by cross-streaking the test strains perpendicularly to RN4220, a strain that is a hyperproducer of β-haemolysin but that does not produce α-haemolysin.[6] Strains SH1000 and SH1001 (the *agr* mutant of SH1000) were used as positive and negative controls, respectively. δ-Haemolytic activity was denoted by an enhanced area of haemolysis at the intersection of the RN4220 streak and the test strain streaks as described.[6] In addition, the δ-haemolysin activity of each strain was semi-quantitatively scored by two investigators involved in this study who were blinded to the isolate numbers, as follows: non-detectable, weak and strong. All experiments were conducted at least three times on separate days.

agrA and RNAIII transcription by quantitative RT–PCR

It is well accepted that transcription from P2 and P3 occurs in a strictly AgrA-dependent manner. However, recent studies have identified that core virulence factors in various *S. aureus* strains depend either on RNAIII or AgrA for regulation.[20] Thus, in the current study, we defined the temporal transcription profiles of both *agrA* and *RNAIII* (as surrogate biomarkers of global *agr* operon activation) by qRT–PCR analyses.[6] In brief,

Table 1. Genotypic characteristics of and vancomycin MICs for study isolates

Strains	CC	*agr* group	SCC*mec*	*spa* type (motif)	Source	Location (reference)	Year	Vancomycin MIC (mg/L)
Clinical isolates								
26997	5	II	IV	2 (TJMBMDMGMK)	blood	USA	2009	1.0
31190	5	II	II	2 (TJMBMDMGMK)	sputum	USA	1996	1.0
29439	8	I	IV	1037 (YHGFMBO)	SSTI	Colombia	2008	1.0
30279	8	I	VIII	1 (YHGFMBQBLO)	SSTI	USA	2010	2.0
31082	8	I	IV	139 (YGFMBLO)	blood	USA	2009	2.0
30568	8	I	IV	7 (YHGCMBQBLO)	blood	USA	2010	2.0
33367	30	III	IV	19 (XKAKAOMQ)	wound	Romania	2005	1.0
17130	30	III	II	16 (WGKAKAOMQQQ)	SSTI	USA	2007	1.0
Reference strains								
36096	5	II	IV	2 (TJMBMDMGMK)	blood	USA	2010	2.0
19069 (USA300-FPR3757)	8	I	IV	1 (YHGFMBQBLO)	abscess	USA	ND	2.0
22033 (WBG10049)	30	III	IV	19 (XKAKAOMQ)	ND	Australia	1999	2.0
21314	45	I	MSSA	XKAKBEMBKB	blood	France	2002	2.0

ND, no data available; SSTI, skin and soft tissue infection.

overnight cultures of *S. aureus* were diluted 100-fold in fresh TSB medium and grown at 37°C in a shaking incubator. Cells were harvested at 2, 3, 4, 8 and 24 h of growth, representing the early-, mid-, late-, post-exponential phases, and the stationary phase, respectively. Quantitative real-time PCR was carried out using an ABI Prism 7000 instrument (Applied Biosystems) and the SYBR green PCR master mix (Applied Biosystems). Reaction mixtures were prepared using the 100 nM primers listed in Table 2. A well-characterized gene, *gyrB*, was used as an internal control.[6,21] Relative target gene expression was calculated as the differences in cycle thresholds (ΔC_T) (*gyrB* C_T – target gene C_T) for all samples.[21] qRT–PCR experiments were performed using at least two biological replicates, with each one tested in triplicate.

Experimental rabbit IE model

It was important to assess the potential translatability of our *in vitro* findings in a realistic and relevant infection model *in vivo*. A catheter-induced model of IE affecting the aortic valve in rabbits was used to study the composite metrics of virulence and responsiveness to vancomycin therapy among the *S. aureus* strains used in the study.[6,22] The animals were maintained in accordance with the American Association for Accreditation of Laboratory Animal Care criteria. The Animal Research Committee (IACUC) of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center approved all the animal studies.

To assess the relative intrinsic virulence of the strains, infected animals ($\sim 10^5$ cfu, representing the ID_{95} inoculum) were euthanized at 24 h after intravenous challenge by a rapid intravenous injection of sodium pentobarbital (200 mg/kg; Abbott Laboratories). The cardiac vegetations, kidneys and spleen were then removed under sterile conditions and quantitatively cultured.[6,22] The tissue densities of *S. aureus* were calculated as the mean $\log_{10}$ cfu/g (±SD).

To assess the response to vancomycin therapy, animals with IE were randomized at 24 h post-infection to receive either no therapy (controls) or vancomycin therapy (15 mg/kg, intravenously, twice daily for 3 days). This is a standard effective dose of vancomycin in the experimental model of IE caused by vancomycin-susceptible staphylococcal strains.[6,22] At 24 h after the last dose of vancomycin, the animals were sacrificed and the target tissues removed and quantitatively cultured as described above.

In vivo population analysis

An *in vivo* population analysis assay was performed to determine whether the *in vivo* emergence of the hVISA phenotype might explain the subsequent impact on either the virulence of the organism or the responsiveness to the antibiotic. A volume of 10 μL of vegetation homogenate was plated directly onto TSA agar plates containing serial concentrations of vancomycin (0.125–8 mg/L) as described above.

In vivo assessment of δ-haemolysin activity

Similar to the hVISA analyses above, it was important to test whether δ-haemolysin activity among the infecting strains was altered during *in vivo* passage. Thus, an *in vivo* assessment of δ-haemolysin production was performed by cross-streaking vegetation homogenates perpendicularly to RN4220 as described above.

Table 2. Primers used in this study

Primer	Sequence (5′–3′)	Reference
gyrB-F	CGCAGGCGATTTTACCATTA	6
gyrB-R	GCTTTCGCTAGATCAAAGTCG	6
RNAIII-F	GCCATCCCAACTTAATAACCA	6
RNAIII-R	TGTTGTTTACGATAGCTTACATGC	6
agrA-F	CGAAGACGATCCAAAACAAAG	48
agrA-R	ATGTTACCAACTGGGTCATGC	48

Statistical analysis

To compare tissue counts of *S. aureus* in vancomycin-treated versus untreated control animals, univariate analyses were performed using the Student's *t*-test. *P* values of <0.05 were considered statistically significant.

Results

In vitro susceptibility to vancomycin

Vancomycin MICs for the study strains were all in the susceptible range as outlined by the CLSI guidelines (Table 1). *In vitro* population analyses of the isolates revealed no hVISA phenotypes [Figure S1 (A1, B1 and C1), available as Supplementary data at *JAC* Online; data not shown for the four reference strains]. Killing curve assays demonstrated no evidence of *in vitro* vancomycin tolerance (data not shown).

In vitro δ-haemolysin activities

A total of two CC5, three CC8, one CC30 and one CC45 isolates produced strong zones of haemolysis due to δ-haemolysin activity (Figure S2). In contrast, the other five strains exhibited weak or non-detectable δ-haemolysin production (Figure S2).

agrA and RNAIII transcriptions

The initiation of transcription of *RNAIII* strictly relies upon the expression of *agrA*, in an *RNAII*-dependent manner. Our data support these observations as the transcription of *agrA* was observed in all strains with *RNAIII* expression except strain 30568 (Figure 1a and b for *agrA* and *RNAIII* expression, respectively). In addition, a rapid increase in *RNAIII* transcription occurred in the post-exponential growth phase, concomitant with a cell-density-dependent expression of the *agr* operon (Figure 1b). More importantly, the seven strains with strong δ-haemolysin activity (one CC5, two CC8 and one CC30 clinical isolate, and three of the four reference strains) exhibited a relatively early onset of *RNAIII* expression, beginning at 2–3 h of growth (Figure 1b). The other five strains had very low-level or no *RNAIII* transcripts detectable before 4 h (Figure 1b). These data indicated that the early onset of *RNAIII* transcription correlated with strong δ-haemolysin production, independent of the CC/agr genotype. As the growth rates for all the study strains were similar, the observed differences in the onset of *RNAIII* expression were not due to differences in growth dynamics (data not shown).

agr locus sequence analyses

We sequenced the entire *agr* locus of the 12 study strains (including *RNAII*, *RNAIII* and their respective promoter regions; Table 3). The results in two CC5-*agr* II strains (exhibiting strong δ-haemolysin activity and an early onset of *RNAIII* activation) revealed identical *agr* locus sequences but these differed from the *agr* sequence reference strain N315 by only one nucleotide between *agrD* and *agrC*. The *agr* sequence of another CC5-*agr* II strain, 31190 (having weak δ-haemolysin activity and a late

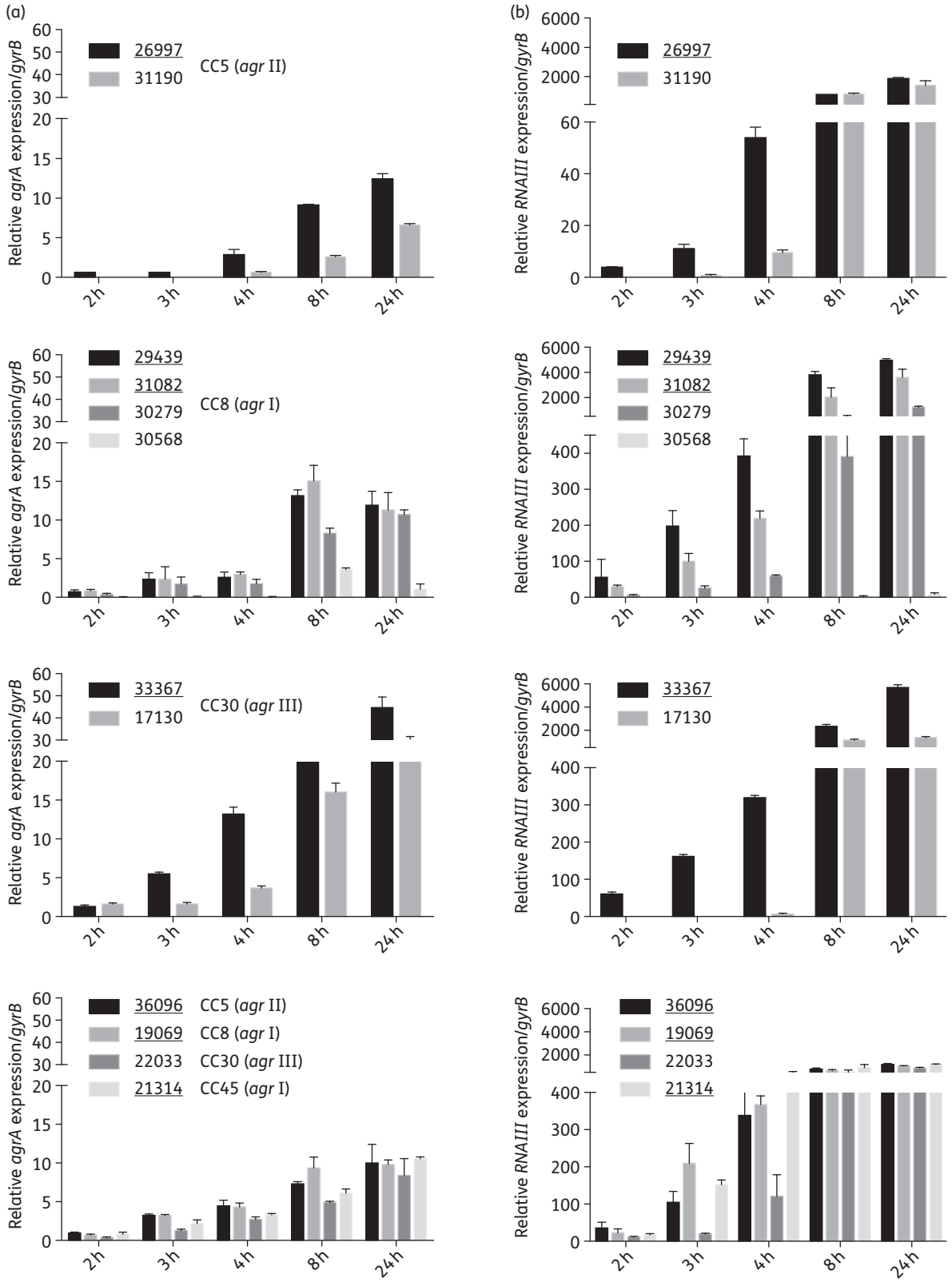

Figure 1. *In vitro* expression of *agrA* (a) and *RNAIII* (b) in all *S. aureus* strains studied at 2, 3, 4, 8 and 24 h of growth. Data were obtained by RT–PCR and relative transcript levels of *agrA* and *RNAIII* represent the mean (+SD) of at least two biological replicates (fold changes versus *gyrB*). Strains with strong δ-haemolysin activity are underlined.

Table 3. *agr* locus sequence, δ-haemolysin and *RNAIII* activation of the study strains

Strains	CC	*agr* group	δ-Haemolysin activity	*RNAIII* activation	*agr* sequence	Reference strains
Clinical isolates						
26997	5	II	strong	early	T→A between *agrD* and *agrC*	N315
31190	5	II	weak	late	T→A between *agrD* and *agrC*, G→A between *hld* and *agrB*,	N315
29439	8	I	strong	early	T→A in *agrC* (F309I)	USA 300
30279	8	I	weak	late	A→G in *RNAIII*, T→A in P3 promoter region, A→T in *agr*B (I171F), T→A in *agrC* (F309I)	USA 300
31082	8	I	strong	early	T→A in P3 promoter region, T→A in *agrC* (F309I)	USA300
30568	8	I	none	none	T→A in P3 promoter region, T→A in *agrC* (F309I)	USA300
33367	30	III	strong	early	*RNAIII* 386A del, *agr*C (R55G)	MRSA 252
17130	30	III	weak	late	none	MRSA 252
Reference strains						
36096	5	II	strong	early	T→A between *agrD* and *agrC*	N315
19069 (USA300-FPR3757)	8	I	strong	early	none	USA300
22033 (WBG10049)	30	III	weak	late	*RNAIII* 386A del, *agrC* (R55G)	MRSA 252
21314	45	I	strong	early	none	CA-347 (CP006044)

onset of expression of *RNAIII*), differed by one nucleotide from these two strains within the intergenic region between *hld* and *agrB*. However, it is not clear whether this *agr* mutational event was related to the different δ-haemolysin activity observed in this strain set. Two CC8-*agr* I (31082 and 30568) and two CC30-*agr* III (33367 and 22033) strains with different δ-haemolysin activities had the same *agr* sequence as their respective reference strains. Taken together, these results indicate that no consensus genetic determinants in the *agr* locus were identified as being predictive of *agr* functionality crossing different CC and *agr* genetic types.

Intrinsic virulence and responsiveness to vancomycin in the IE model

The intrinsic virulence of the study strains in the IE model (based on achievable target-tissue counts of *S. aureus*) was similar among the study isolates (Figure 2). Therefore, there was no obvious relationship between the above *agr* metrics, CC types and intrinsic virulence of the strains studied in this model of infection.

In contrast, our data revealed a direct correlation between temporal *agr RNAIII* expression *in vitro* and vancomycin therapy *in vivo*. For example, the vancomycin therapy of animals infected with the five strains that exhibited a late onset of *RNAIII* and weak or no δ-haemolysin activity *in vitro* (clinical isolates 31190, 30279, 30568 and 17130, and reference strain 22033) resulted in uniform and highly significant reductions in MRSA counts in all the target tissues (Figure 2). Thus, we observed a $\geq$5 $\log_{10}$ cfu reduction in the vegetation counts and $\geq$3 $\log_{10}$ cfu reduction in the kidney and spleen counts compared with the respective untreated control groups ($P < 0.0001$ for all strains and all three target tissues). In contrast, animals with IE caused by strains with strong δ-haemolysin activity and an early onset of *RNAIII* transcription showed one of two outcomes: (i) no response to vancomycin treatment, with residual target-tissue MRSA densities similar to those in the respective untreated controls (the 33367 clinical isolate and the 36096 reference strain; Figure 2); or (ii) a $<$3 $\log_{10}$ cfu reduction in target-tissue counts (clinical isolates 26997, 29439 and 31082, and reference strains 19069 and 21314; Figure 2). Importantly, there was no significant difference in the weight of the vegetations between the groups (data not shown). Therefore, the microbiological difference in vegetation counts was not impacted by the differences in structure or size of the vegetations.

In vivo population analyses

Figure S1 (A2, B2, B3 and C2) shows the *in vivo* population analyses from vegetation homogenates of the study clinical isolates surviving vancomycin therapy in the IE model (data not shown for the four reference strains). No subpopulations that were heteroresistant to vancomycin were observed in the vegetations from any animals (Figure S1; data not shown for the control animals without treatment). We were unable to test for potential hVISA phenotypes in strains highly susceptible to vancomycin therapy in the IE model due to the low residual bacterial counts of the vegetations ($<$3 $\log_{10}$ cfu/g).

In vivo δ-haemolysin activity

No changes in profiles of δ-haemolysin activity were observed in any isolates after passage through the IE model (Figure S2; data not shown for the four reference strains).

Discussion

S. aureus is a highly adaptable bacterium capable of dynamic changes in its virulence and resistance phenotypes in the face

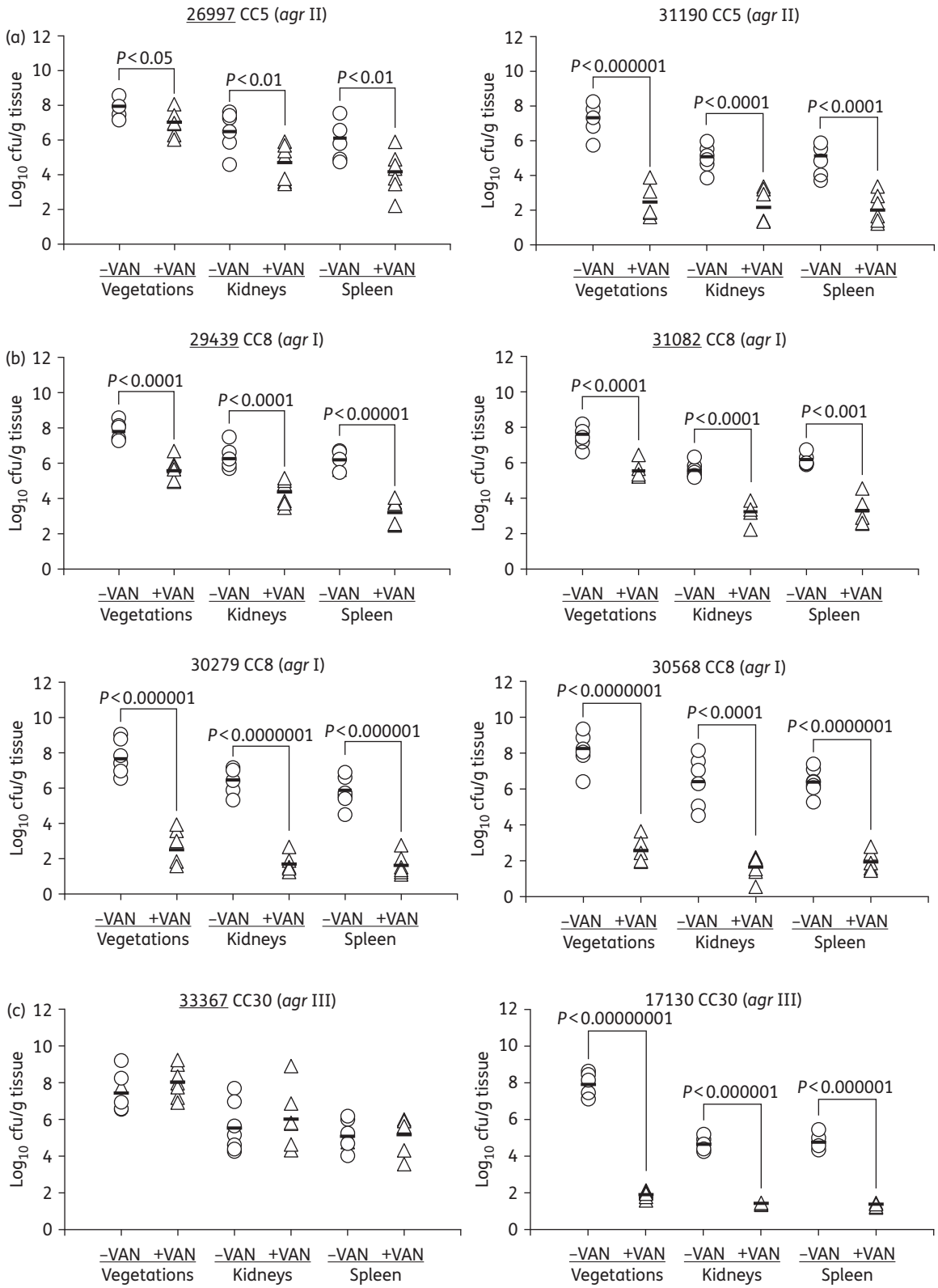

Figure 2. *S. aureus* densities in the target tissues in the IE model in the presence versus absence of vancomycin therapy for 3 days (panels a, b and c represent clinical CC5, CC8 and CC30 *S. aureus* strains, respectively, and panel d represents four reference strains). Each dot represents one rabbit and the horizontal black bars indicate the means of the observations. Strains with strong δ-haemolysin activities are underlined. VAN, vancomycin.

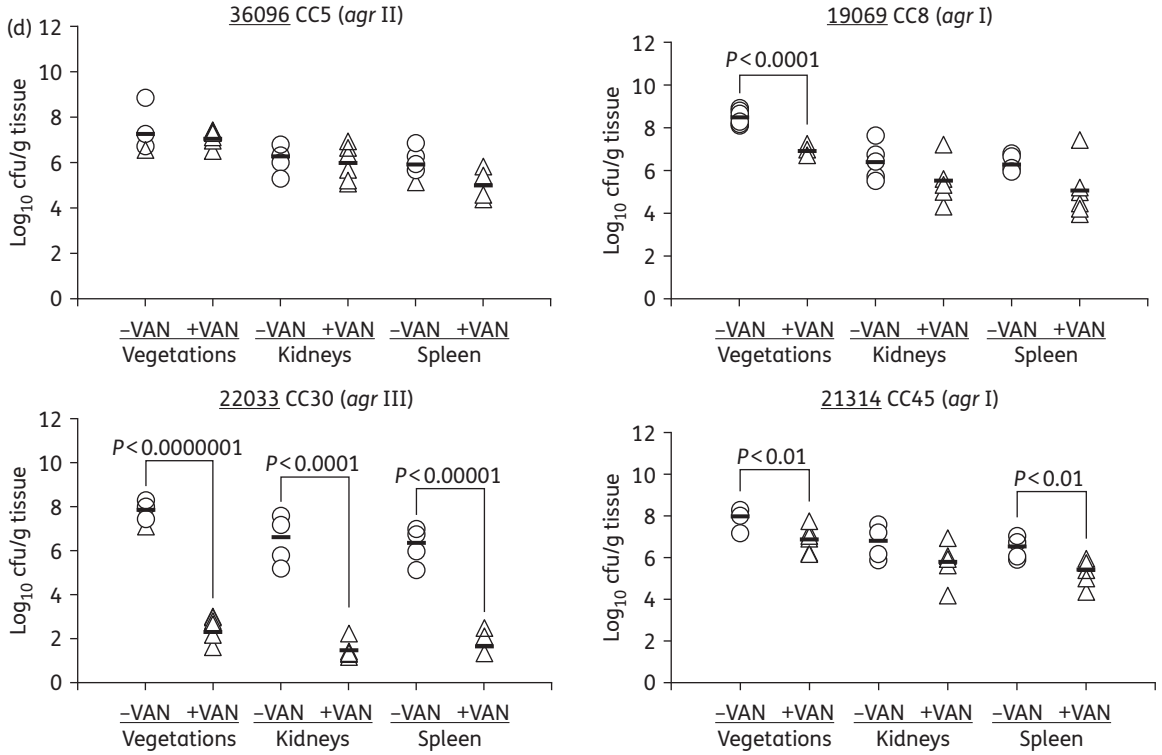

Figure 2. *Continued*

of exposure to host defences or administered antibiotics. As a result of such capabilities, 'persistent' endovascular MRSA infections frequently occur despite seemingly appropriate antibiotic therapy. This reality represents a daunting and increasingly life-threatening clinical syndrome. The fundamental phenotypic and genotypic virulence and resistance 'signatures' of *S. aureus* that drive such persistent clinical outcomes are not well defined. Thus, the identification of potential biomarkers that specifically characterize persistent *S. aureus* strains is urgently needed. The current study was designed to contribute new insights that might help to address this void in knowledge.

The *agr* operon plays an important and complex role in the pathogenesis of MRSA infections.[9,23,24] In addition, polymorphisms in *agrD* and *agrC* have been utilized to genotypically define the major *S. aureus agr* groups (I–IV).[25–27] Several previous studies have suggested an association of specific *agr* groups with distinct clinical syndromes involving *S. aureus*.[25,26] For example, *agr* group II has been associated with a poor response to vancomycin therapy in patients with MRSA infections.[28] Of note, such linkage of the *agr* group II genotype to suboptimal treatment outcomes has not been verified in more recent reports using a rabbit model of IE.[6] Interestingly, in addition to *agr* genotypes, four CC types (CC5, CC8, CC30 and CC45) are reported to predominate in serious *S. aureus* infections.[29,30] Moreover, distinct CC types show a differing potential to cause invasive disease.[31] For instance, CC5 and CC30 have been reported to be associated with more severe infections.[11] Furthermore, Miller *et al.*[32] recently correlated the CC22 genotype with cases of IE. Conversely, Feil *et al.*[33] reported that disease isolates were equally represented across all CC types, suggesting that the link between CC genotype and the propensity to cause specific disease syndromes is at best inconsistent. The above examples illustrate how the genotypic and phenotypic plasticity of *S. aureus* is likely to afford a variety of survival strategies and advantages in distinct host defence settings. Thus, rather than seeking a single gene or distinct host phenotype that enables persistence, our work seeks to identify and validate more integrated signals of adaptive responses in *S. aureus* that are associated with virulence and antimicrobial resistance.

Vancomycin has remained a stalwart for the treatment of invasive MRSA infections. We recently studied the relationship of *agr RNAIII* expression with vancomycin treatment outcomes in experimental IE due to 10 clinical MRSA strains with two distinct genetic backgrounds (CC5-*agr* II and CC45-*agr* I). These investigations demonstrated that early-onset *agr RNAIII* activation correlated with failures of vancomycin treatment in this model.[6] Therefore, our current studies were designed to further investigate the question of whether this putative correlation between the temporal *agr RNAIII* activation profiles and the outcome of antibiotic treatment is CC-*agr* type specific or more generic across CC-*agr* types. In this regard, our strategy was strengthened by the use of clinical MRSA isolates representing the most common CC-*agr* types (CC5-*agr* II, CC8-*agr* I and CC30-*agr* III) in comparison with four respective *S. aureus* reference strains.

Several key insights emerged from this study. We found that the overall temporal transcription of *RNAIII* was strain dependent but CC/*agr* type independent. Most importantly, consistent with our previous observation,[6] we found that early-onset *agr RNAIII* activation (beginning at 2–3 h of growth) significantly correlated with reduced vancomycin efficacy in experimental IE. The exact mechanism(s) by which early *agr* activation provides the organism

with reduced vancomycin susceptibility *in vivo* in this model is yet to be defined. However, in this regard, we have recently confirmed that strains exhibiting early *RNAIII* activation (which are vancomycin non-responders in the IE model) differed substantially from the strains showing later *RNAIII* expression (vancomycin responders in the same model) in terms of: (i) lower vancomycin binding; (ii) increased intrinsic biofilm formation; (iii) higher survival in the presence of vancomycin within biofilms in the presence or absence of catheters; (iv) enhanced biofilm formation during vancomycin exposure below the MIC; (v) significantly greater damage to endothelial cells;[19,34] and (vi) reduced killing by certain host defence antimicrobial peptides.[35] Each of these differences may contribute individually or in combination to the strain-specific vancomycin treatment outcomes in the IE model. Importantly, we performed an *in vitro* growth assay and found that all the study strains had similar growth rates. In addition, all the study isolates had similar fitness profiles in the IE model: (i) all strains had the same ID_{95} for the induction of IE (10^5 cfu/animal); and (ii) the intrinsic virulence of the study strains in the IE model, based on achievable target-tissue counts of *S. aureus*, was similar among the study isolates (Figure 2). These data indicate that the observed differences in the onset of *agr RNAIII* expression and treatment outcomes were not due to differences in *in vivo* growth dynamics. In contrast to our observations, other studies have suggested that *agr* dysfunction can be associated with decreased vancomycin efficacy and persistent bacteraemia in MRSA infections.[5,27,36–38] For instance, Fowler *et al.*[5] reported that δ-haemolysin activity was less common in persistent versus resolving MRSA isolates. However, these differences did not reach statistical significance.[5] In addition, these conflicting findings may reflect geographical or other epidemiological patterns. Of note, it has been reported that even though the lack of δ-haemolysin production is a phenotypic marker of *agr* dysfunction,[5,39,40] its absence does not necessarily correlate with the absence of *RNAIII* transcripts.[41]

Paralleling our experimental model outcomes, clinical treatment failures with vancomycin occur fairly commonly even with 'vancomycin-susceptible' *S. aureus* strains.[42] Some studies have suggested that an *in vivo* evolution of the hVISA phenotype during therapy can be responsible for such clinical failures.[43] However, in the current study, we could not confirm hVISA development *in vivo* during vancomycin therapy.

Consistent with previous studies from our laboratories[6] and others,[44–46] we found no common or consensus SNPs within the *agr* locus that predicted its temporal activation profiles and/or functionality *in vitro*, or vancomycin responsiveness in experimental IE crossing different CC/*agr* types. In support of our findings, other investigations have also found no differences in the *agr* sequence between clinical pairs of parental vancomycin-susceptible *S. aureus* strains and hVISA/VISA strains evolving during antibiotic-persistent infection.[45,46] Taken together, these findings suggest that strain-specific gain-of-function or loss-of-function readouts of genetic determinants in the *agr* locus cannot yet be linked to *agr* functionality and vancomycin treatment outcomes. This observation also implies that genotypic factors beyond or interacting with the *agr* locus may play a role in adaptive response phenotypes with respect to virulence and antibiotic resistance outcomes *in vivo*.

Our investigations have several limitations. For example, hVISA strains were not studied in our model; such strains may be very important as they have been associated with high frequencies of treatment failure and persistent bacteraemia.[43,47] In addition, additional *S. aureus* isolates from patients with known endovascular infections should be evaluated to better understand the precise factors responsible for persistent outcomes in this clinical context. Moreover, based on current findings, we do not know whether the relationship between the *RNAIII* activation profiles and the outcome of antibiotic treatment is 'vancomycin specific' or more broad range against other antimicrobials. Although this is beyond the scope of the present study, future studies should examine this potential correlation.

In summary, we have demonstrated that: (i) early *agr RNAIII* activation, although not 'causal',[6] is a 'biomarker' or 'signature' predictive of an antibiotic-persistent outcome; and (ii) factors outside the *agr* locus are responsible for the genetic linkage between temporal *agr RNAIII* activation and the outcome of vancomycin treatment in the IE model. The exact mechanism(s) that correlate with the failure of vancomycin treatment remain to be fully elucidated. Studies including whole-genome sequencing, susceptibilities to the peptides involved in host defences against antimicrobial agents (e.g. platelet microbicidal proteins and human neutrophil peptide 1), biofilm formation, endothelial cell damage, vancomycin binding, etc. are in progress in our laboratories to better define these mechanism(s).

Funding

This work was supported in part by grants from the National Institutes of Health (grants R21AI097657 to Y. Q. X., RO1AI-39108 to A. S. B. and AI111 to M. R. Y.); and the U.S. Department of Defense (grant W81XWH-12-2-0101 to M. R. Y.).

Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Fowler VG Jr, Miro JM, Hoen B *et al*. *Staphylococcus aureus* endocarditis: a consequence of medical progress. *JAMA* 2005; **293**: 3012–21.

2 Wisplinghoff H, Bischoff T, Tallent SM *et al*. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* 2004; **39**: 309–17.

3 Naber CK. *Staphylococcus aureus* bacteremia: epidemiology, pathophysiology, and management strategies. *Clin Infect Dis* 2009; **48** Suppl 4: S231–7.

4 Khatib R, Johnson LB, Fakih MG *et al*. Persistence in *Staphylococcus aureus* bacteremia: incidence, characteristics of patients and outcome. *Scand J Infect Dis* 2006; **38**: 7–14.

5 Fowler VG Jr, Sakoulas G, McIntyre LM *et al*. 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* 2004; **190**: 1140–9.

6 Seidl K, Chen L, Bayer AS *et al*. Relationship of agr expression and function with virulence and vancomycin treatment outcomes in experimental endocarditis due to methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2011; **55**: 5631–9.

7 Cheung AL, Bayer AS, Zhang G *et al*. Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. *FEMS Immun Med Microbiol* 2004; **40**: 1–9.

8 Novick RP. Pathogenicity factors and their regulation. In: Fischett VA, Novick RP, Ferretti JJ *et al*., eds. *Gram-positive Pathogens*. Washington, DC: American Society for Microbiology, 2000; 392–407.

9 Novick RP, Geisinger E. Quorum sensing in staphylococci. *Annu Rev Genet* 2008; **42**: 541–64.

10 Traber K, Novick R. A slipped-mispairing mutation in AgrA of laboratory strains and clinical isolates results in delayed activation of *agr* and failure to translate δ- and α-haemolysins. *Mol Microbiol* 2006; **59**: 1519–30.

11 Fowler VG Jr, Nelson CL, McIntyre LM *et al*. Potential associations between hematogenous complications and bacterial genotype in *Staphylococcus aureus* infection. *J Infect Dis* 2007; **196**: 738–47.

12 McCalla C, Smyth DS, Robinson DA *et al*. Microbiological and genotypic analysis of methicillin-resistant *Staphylococcus aureus* bacteremia. *Antimicrob Agents Chemother* 2008; **52**: 3441–3.

13 Nienaber JJ, Sharma Kuinkel BK, Clarke-Pearson M *et al*. Methicillin-susceptible *Staphylococcus aureus* endocarditis isolates are associated with clonal complex 30 genotype and a distinct repertoire of enterotoxins and adhesins. *J Infect Dis* 2011; **204**: 704–13.

14 Fowler VG Jr, Boucher HW, Corey GR *et al*. Daptomycin versus standard therapy for bacteremia and endocarditis caused by *Staphylococcus aureus*. *N Engl J Med* 2006; **355**: 653–65.

15 Lina G, Boutite F, Tristan A *et al*. Bacterial competition for human nasal cavity colonization: role of Staphylococcal *agr* alleles. *Appl Environ Microbiol* 2003; **69**: 18–23.

16 Chen L, Mediavilla JR, Oliveira DC *et al*. Multiplex real-time PCR for rapid staphylococcal cassette chromosome *mec* typing. *J Clin Microbiol* 2009; **47**: 3692–706.

17 Mathema B, Mediavilla J, Kreiswirth BN. Sequence analysis of the variable number tandem repeat in *Staphylococcus aureus* protein A gene: *spa* typing. *Methods Mol Biol* 2008; **431**: 285–305.

18 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: M7-A9*. CLSI, Wayne, PA, USA, 2012.

19 Abdelhady W, Bayer AS, Seidl K *et al*. Reduced vancomycin susceptibility in an *in vitro* catheter-related biofilm model correlates with poor therapeutic outcomes in experimental endocarditis due to methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents Chemother* 2013; **57**: 1447–54.

20 Queck SY, Jameson-Lee M, Villaruz AE *et al*. RNAIII-independent target gene control by the *agr* quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. *Mol Cell* 2008; **32**: 150–8.

21 Abdelhady W, Bayer AS, Seidl K *et al*. Impact of vancomycin on *sarA*-mediated biofilm formation: role in persistent endovascular infections due to methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* 2014; **209**: 1231–40.

22 Xiong YQ, Fowler VG, Yeaman MR *et al*. Phenotypic and genotypic characteristics of persistent methicillin-resistant *Staphylococcus aureus* bacteremia *in vitro* and in an experimental endocarditis model. *J Infect Dis* 2009; **199**: 201–8.

23 Cheung GY, Wang R, Khan BA *et al*. Role of the accessory gene regulator *agr* in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis. *Infect Immun* 2011; **79**: 1927–35.

24 Traber KE, Lee E, Benson S *et al*. *agr* function in clinical *Staphylococcus aureus* isolates. *Microbiol* 2008; **154**: 2265–74.

25 Ji G, Beavis R, Novick RP. Bacterial interference caused by autoinducing peptide variants. *Science* 1997; **276**: 2027–30.

26 Jarraud S, Lyon GJ, Figueiredo AM *et al*. Exfoliatin-producing strains define a fourth *agr* specificity group in *Staphylococcus aureus*. *J Bacteriol* 2000; **182**: 6517–22.

27 Sakoulas G, Eliopoulos GM, Moellering RC Jr *et al*. Accessory gene regulator (*agr*) locus in geographically diverse *Staphylococcus aureus* isolates with reduced susceptibility to vancomycin. *Antimicrob Agents Chemother* 2002; **46**: 1492–502.

28 Moise-Broder PA, Sakoulas G, Eliopoulos GM *et al*. Accessory gene regulator group II polymorphism in methicillin-resistant *Staphylococcus aureus* is predictive of failure of vancomycin therapy. *Clin Infect Dis* 2004; **38**: 1700–5.

29 Wertheim HF, Melles DC, Vos MC *et al*. The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis* 2005; **5**: 751–62.

30 Enright MC, Robinson DA, Randle G *et al*. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *PNAS* 2002; **99**: 7687–92.

31 Brueggemann AB, Griffiths DT, Meats E *et al*. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis* 2003; **187**: 1424–32.

32 Miller CE, Batra R, Cooper BS *et al*. An association between bacterial genotype combined with a high-vancomycin minimum inhibitory concentration and risk of endocarditis in methicillin-resistant *Staphylococcus aureus* bloodstream infection. *Clin Infect Dis* 2012; **54**: 591–600.

33 Feil EJ, Cooper JE, Grundmann H *et al*. How clonal is *Staphylococcus aureus*? *J Bacteriol* 2003; **185**: 3307–16.

34 Seidl K, Bayer AS, McKinnell JA *et al*. *In vitro* endothelial cell damage is positively correlated with enhanced virulence and poor vancomycin responsiveness in experimental endocarditis due to methicillin-resistant *Staphylococcus aureus*. *Cell Microbiol* 2011; **13**: 1530–41.

35 Seidl K, Bayer AS, Fowler VG Jr *et al*. Combinatorial phenotypic signatures distinguish persistent from resolving methicillin-resistant *Staphylococcus aureus* bacteremia isolates. *Antimicrobial Agents Chemother* 2011; **55**: 575–82.

36 Sakoulas G, Moellering RC Jr, Eliopoulos GM. Adaptation of methicillin-resistant *Staphylococcus aureus* in the face of vancomycin therapy. *Clin Infect Dis* 2006; **42** Suppl 1: S40–50.

37 Sakoulas G, Eliopoulos GM, Fowler VG Jr *et al*. 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* 2005; **49**: 2687–92.

38 Schweizer ML, Furuno JP, Sakoulas G *et al*. Increased mortality with accessory gene regulator (*agr*) dysfunction in *Staphylococcus aureus* among bacteremic patients. *Antimicrob Agents Chemother* 2011; **55**: 1082–7.

39 Moise PA, Forrest A, Bayer AS *et al*. Factors influencing time to vancomycin-induced clearance of nonendocarditis methicillin-resistant *Staphylococcus aureus* bacteremia: role of platelet microbicidal protein killing and *agr* genotypes. *J Infect Dis* 2010; **201**: 233–40.

40 Sakoulas G, Eliopoulos GM, Moellering RC Jr *et al*. *Staphylococcus aureus* accessory gene regulator (*agr*) group II: is there a relationship to the development of intermediate-level glycopeptide resistance? *J Infect Dis* 2003; **187**: 929–38.

41 Wright JS 3rd, Traber KE, Corrigan R *et al*. The *agr* radiation: an early event in the evolution of staphylococci. *J Bacteriol* 2005; **187**: 5585–94.

42 Moise PA, Schentag JJ. Vancomycin treatment failures in *Staphylococcus aureus* lower respiratory tract infections. *Int J Antimicrob Agents* 2000; **16** Suppl 1: S31–4.

43 Moreillon P, Bizzini A, Giddey M *et al*. Vancomycin-intermediate *Staphylococcus aureus* selected during vancomycin therapy of

experimental endocarditis are not detected by culture-based diagnostic procedures and persist after treatment arrest. *J Antimicrob Chemother* 2012; **67**: 652–60.

44 Howden BP, Johnson PD, Ward PB *et al*. Isolates with low-level vancomycin resistance associated with persistent methicillin-resistant *Staphylococcus aureus* bacteremia. *Antimicrob Agents Chemother* 2006; **50**: 3039–47.

45 Howden BP, Smith DJ, Mansell A *et al*. Different bacterial gene expression patterns and attenuated host immune responses are associated with the evolution of low-level vancomycin resistance during persistent methicillin-resistant *Staphylococcus aureus* bacteraemia. *BMC Microbiol* 2008; **8**: 39.

46 Park C, Shin NY, Byun JH *et al*. Downregulation of RNAIII in vancomycin-intermediate *Staphylococcus aureus* strains regardless of the presence of *agr* mutation. *J Med Microbiol* 2012; **61**: 345–52.

47 Howden BP, Davies JK, Johnson PD *et al*. Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clin Microbiol Rev* 2010; **23**: 99–139.

48 Burnside K, Lembo A, de Los Reyes M *et al*. Regulation of hemolysin expression and virulence of *Staphylococcus aureus* by a serine/threonine kinase and phosphatase. *PLoS One* 2010; **5**: e11071.