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agr RNAIII divergently regulates glucose-induced biofilm formation in clinical isolates of *Staphylococcus aureus*

Leonardo Rocchetto Coelho,† Raquel Rodrigues Souza,† Fabienne Antunes Ferreira, Márcia Aparecida Guimarães, Bernadete Teixeira Ferreira-Carvalho and Agnes Marie Sá Figueiredo

Correspondence
Agnes Marie Sá Figueiredo
agnes@micro.ufrj.br

Universidade Federal do Rio de Janeiro, Instituto de Microbiologia Professor Paulo de Góes, Av. Carlos Chagas Filho 373 – CCS – Bl I, Cidade Universitária, 21941590 Rio de Janeiro, Brazil

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Staphylococcus aureus is an important nosocomial and community-acquired pathogen. Hospital infections are frequently complicated by the ability of bacteria to form biofilms on different surfaces. The development of bacterial films on medical indwelling devices, such as prostheses, often requires surgical procedures to remove the contaminated implant. Indeed, biofilm formation on central endovenous catheters is a major cause of primary bacteraemia in hospitals. The modulation of virulence factors in *S. aureus* is orchestrated by a number of global regulators including *agr* RNAIII. To improve our understanding of the role of the *agr* quorum-sensing system in biofilm formation by *S. aureus*, we constructed a number of *agr*-null mutants, derived from contemporary clinical isolates. Analysis of these mutants indicates that *agr* has a significant impact on biofilm development for most of the isolates tested. Our data show that RNAIII can control both biofilm formation and accumulation. The *agr* effect included both up- and downregulation of biofilms, even for isolates within the same lineage, corroborating the hypothesis that the mechanisms involved in *S. aureus* biofilms are complex and probably multifactorial.

INTRODUCTION

Staphylococcus aureus biofilm formation is an important factor in the pathogenesis of central venous catheter-associated bacteraemia and infections related to the use of medical prostheses (Vuong *et al.*, 2000). Despite this knowledge, the composition of *S. aureus* biofilms and the associated regulatory network for their production have not been fully clarified thus far. The *ica* locus, which encodes proteins involved in the biosynthesis of the polysaccharide intercellular adhesion or poly-*N*-acetylglucosamine (PIA/PNAG) is present in the genome of both *S. aureus* and *Staphylococcus epidermidis*. Deletion of *ica* in *S. aureus* strain ATCC 35556 has been shown to inhibit glucose-induced biofilm development on polystyrene surfaces, under static conditions (Cramton *et al.*, 1999). However, *ica*-independent mechanisms of biofilm formation have also been reported, and recently it was shown that a SasG-expressing mutant of *S. aureus* strain SH1000 formed a glucose-induced biofilm on the surface of fibronectin- or fibrinogen-coated plates (Corrigan *et al.*, 2007). Studies by O'Neill *et al.* (2007) indicated that glucose-induced biofilm formation in meticillin-resistant *S. aureus* (MRSA) was mainly *ica*-independent and probably mediated by protein(s). More recently, the same researchers showed that FnbpA and B were involved in glucose-induced biofilm development of meticillin-susceptible *S. aureus* (MSSA) and MRSA clinical isolates, under static and flow conditions (O'Neill *et al.*, 2008).

SarA (a global transcriptional regulator of *S. aureus*) was found to be a positive regulator of both *ica*-dependent and independent biofilm formation (Valle *et al.*, 2003; Beenken *et al.*, 2003; Toledo-Arana *et al.*, 2005; O'Neill *et al.*, 2007). It was shown that mutation in *arlRS* in *S. aureus* strain 15981 led to an increased biofilm accumulation even when *ica* was deleted. The elimination of the major *S. aureus* quorum-sensing (QS) locus, *agr*, had no effect on *ica*-independent biofilm repressed by ArlRS. Indeed, no difference was observed when those studies were carried out under static or flow conditions (Toledo-Arana *et al.*, 2005). Beenken and colleagues, studying 13 *agr*-null constructs of *S. aureus*, proposed that mutation in *agr* had only a modest impact on the biofilm development induced by both glucose and sodium chloride on the surface of fibronectin-precoated plates. Nevertheless, they

†These authors contributed equally to this work.

Abbreviations: AIP, Agr autoinducing peptide; BEC, Brazilian epidemic clone; BU, biofilm unit; CLSM, confocal laser scanning microscopy; CS, conditioned supernatant; MRSA, meticillin-resistant *S. aureus*; MSSA, meticillin-susceptible *S. aureus*; WT, wild-type.

also reported that the only exception was with *S. aureus* strain RN6390B, which regularly formed a biofilm only after *agr* deletion (Beenken *et al.*, 2003). Similarly, it has been demonstrated by others that removing *agr* from *S. aureus* strains 15981 and V329 had no impact on biofilm formation in the presence of glucose, under flow conditions (Valle *et al.*, 2003). In spite of this, Yarwood *et al.* (2004) showed that the biofilm modulation promoted by *agr* in *S. aureus* could diverge under different environmental conditions. In fact, other studies found biofilm was enhanced in the *agr*-null strain RN6911 and in the *agrC*-transposon mutant *mut6*, suggesting that *agr* downregulates biofilm formation induced by glucose on the surface of polystyrene plates (Vuong *et al.*, 2000).

It is important to note that most of the studies cited here were carried out with a small number of constructs or with NCTC 8325-4 derivatives, including RN6911. More recently, using multiple *agr* mutants, 1 % glucose induction and microtitre plate assays for biofilm evaluation, it was concluded that *agr* elimination increased *ica*-independent biofilm formation in five constructs and had no significant effect on the 16 others (O'Neill *et al.*, 2007). Using an *in vivo* model to study biofilm, Balaban *et al.* (2007) reported that either TRAP$^-$ or *agr*-null constructs derived from strain 8325-4 were deficient in biofilm formation. Thus, these authors suggested that RIP (an RNAIII-inhibiting peptide) could be used as a therapeutic agent to prevent biofilm development. In contrast, Tsang *et al.* (2007) found that *traP* mutations in *S. aureus* UAMS-1 and USA 300 did not affect *agr* or biofilm formation induced by glucose on the surface of polystyrene, under static conditions.

Clearly, the role played by *agr* on *S. aureus* biofilms is at best variable, and remains to be better understood. To address this, we analysed 12 MRSA and MSSA *agr*-null constructs derived from contemporary clinical isolates to further investigate the influence of the RNAIII transcript (presence or absence) on biofilm formation/accumulation by *S. aureus*. In addition, we examined biofilm development in a variant of MRSA ST239-SCC*mec*IIIA, which displays natural *sarA*/*agr* attenuation.

METHODS

Bacterial strain, plasmid and construction. *S. aureus* strain RN450 was used for phage propagation (Kreiswirth *et al.*, 1983). *S. aureus* strain RN6911(Δ*agr*::*tetM*) is a derivative of strain RN6390B (Nesin *et al.*, 1990; Novick *et al.*, 1993). The *agr*-null constructs were obtained by allele replacement of the entire *agr* locus. Mutation Δ*agr*::*tetM* was transduced from strain RN6911 into MSSA or MRSA clinical isolates using phage 80α (Novick, 1967). The presence of RNAIII transcript in all wild-types (WT) was confirmed by RT-PCR and Northern blotting. Experiments of RNAIII trans-complementation were carried out by introducing pRN6848 (pRN5548::*rnaIII-cat*) into the *agr*-null MRSA or MSSA, also through 80α phage transduction (Novick, 1967; Novick *et al.*, 1993). The transductants were selected on antibiotic-supplemented plates (*agr*-null with 5 μg tetracycline ml^{-1} and *rnaIII*-complemented mutants with 10 μg chloramphenicol ml^{-1}). The *agr* deletions and *rnaIII* complementations were confirmed by PCR, dot-blot and Northern-blot assays using an *rnaIII*-specific probe. The genetic backgrounds of the mutants and respective WT were checked by PFGE, as described below. The MRSA isolates studied belonged to hospital- or community-associated international MRSA lineages currently isolated on the American continent. Strains RN6911 and RN450, and pRN6848, were kindly provided by Richard Novick, Skirball Institute of Biomolecular Medicine, NY, USA. The WT isolates and the corresponding isogenic mutants are listed in Table 1. In addition, some isolates of MRSA lineage ST239-SCC*mec*IIIA (Brazilian epidemic clone; BEC) were also included in this study. All bacterial strains and constructs were stored in 12 % glycerol at −70 °C.

Molecular characterization. The SCC*mec* typing of the MRSA clinical isolates was performed as described by Oliveira & de Lencastre (2002). PFGE of the *Sma*I-fragmented DNA was carried out as described by Teixeira *et al.* (1995). The criterion used for classifying the PFGE patterns was suggested by Tenover *et al.* (1995), and was based on comparisons with the PFGE patterns displayed by representatives of international MRSA lineages (Ribeiro *et al.*, 2007). Experiments of multilocus sequence typing (MLST) were also performed and the sequence type (ST) was assigned with reference to the MLST database (http://www.mlst.net).

PCR. Specific primers for *rnaIII* (Novick *et al.*, 1993), *sarA* (5′-ggcaaatgtatcgagcaagatg-3′ and 5′-gtatcatctatcaaacttcacc-3′) and 16S *rrna* (5′-aacgcattaagcactccgc-3′ and 5′-gtgtgtagcccaaatcataa-3′) were used for PCR amplifications and probe preparations in the experiments involving DNA–DNA or DNA–RNA hybridizations. DNA preparations were obtained by phenol extraction and used in standard PCRs (Sambrook *et al.*, 1989). The annealing temperature for all PCR programmes was 55 °C. The primers for *sarA* and 16S *rrna* were designed based on conserved regions of the respective sequences deposited at GenBank. Multiple alignments, using CLUSTAL W (Thompson *et al.*, 1994), were performed to check for *in silico* primer specificity.

RT-PCR. Total RNA was obtained using the RNeasy Mini kit (Qiagen). Purified RNA preparations were quantified using GeneQuant RNA/DNA Calculator (GE Healthcare). Prior to performing RT-PCR, 1 μg of the RNA preparation was treated with 1 U amplification-grade DNase I as recommended for RT-PCR (Invitrogen). All DNase reactions were controlled by PCR in the absence of reverse transcriptase. RT-PCR (SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase; Invitrogen) was performed as recommended by the manufacturer using 1 μg of the template RNA. cDNA synthesis was carried out at 50 °C for 30 min, denatured at 94 °C for 2 min, and amplified in 40 cycles: 94 °C for 15 s (denature), 55 °C for 30 s (anneal) and 68 °C for 30 s (extend); followed by a final extension at 68 °C for 5 min (one-step). The *rnaIII*-specific primers used have been described previously (Novick *et al.*, 1993).

Dot-blotting. Genomic DNA of the *agr*-null mutants and of the *rnaIII*-complemented constructs was denatured by boiling and spotted onto the surface of a nylon membrane. The membrane (Hybond-N+; GE Healthcare Bio-Sciences) was fixed by baking, then hybridized with labelled *rnaIII* probe and detected using Alkphos Direct Labelling with CDP STAR (GE Healthcare), as recommended by the manufacturer.

Northern blotting. Ten nanograms of the total RNA, prepared as above, were run in an electrophoresis system in a 1.2 % RNA-agarose gel, as recommended by the RNeasy kit manufacturer (Qiagen). The RNA was transferred to a nylon membrane (Hybond-N+, GE Healthcare) using vacuGene XL (GE Healthcare). Hybridization and detection of chemoluminescence signals were carried out using the kit

Table 1. Effect of *agr* on biofilm formation of MRSA and MSSA isolates

Strain (related lineage)	Genetic characteristic	BU*	Biofilm phenotype	*agr* regulation
MSSA				
HC474	*mecA*	1.12	Strong	Upregulation
MHC474	Δ*agr*::*tetM*	0.36	Weak	($P<0.001$)
CMHC474	Δ*agr*::*tetM*, p*bla-rnaIII*	0.95	Strong	
HC474 + CS†		0.39	Weak	
MSSA				
HC642	*mecA*	1.35	Strong	Upregulation
MHC642	Δ*agr*::*tetM*	0.66	Moderate	($P<0.01$)
MRSA (USA 100)				
NY17859	(ST5-SCC*mec*II)	2.81	Strong	Upregulation
MNY17859	Δ*agr*::*tetM*	0.37	Weak	($P<0.001$)
CA-MRSA (USA 400)				
WB81	(ST1-SCC*mec*IV)	1.01	Strong	Upregulation
MWB81	Δ*agr*::*tetM*	0.31	Weak	($P<0.01$)
MRSA (BEC)				
BMB9393	(ST239-SCC*mec*IIIA)	2.78	Strong	Upregulation
BMB9393 + CS		0.78	Moderate	($P<0.001$)
MRSA (BEC)				
GV69	(ST239-SCC*mec*IIIA)	0.90	Moderate	Upregulation
GV69 + CS		0.48	Moderate	($P<0.05$)
MRSA (USA 100)				
NY17896	(ST5-SCC*mec*II)	0.25	Weak	Neutral
MNY17896	Δ*agr*::*tetM*	0.31	Weak	No significance
MRSA (USA 100)				
NY22735	(ST5-SCC*mec*II)	0.38	Weak	Neutral
MNY22735	Δ*agr*::*tetM*	0.29	Weak	No significance
MSSA				
HC410	*mecA*	0.16	Non-producer	Neutral
MHC410	Δ*agr*::*tetM*	0.15	Non-producer	No significance
MSSA				
HC569	*mecA*	0.35	Weak	Downregulation
MHC569	Δ*agr*::*tetM*	0.69	Moderate	($P<0.01$)
CMHC569	Δ*agr*::*tetM*, p*bla-rnaIII*	0.42	Weak	
HC569 + CS		0.62	Moderate	
MRSA (USA 100)				
NY19335	(ST5-SCC*mec*II)	0.33	Weak	Downregulation
MNY19335	Δ*agr*::*tetM*	1.28	Strong	($P<0.001$)
CMNY19335	Δ*agr*::*tetM*, p*bla-rnaIII*	0.3	Weak	
NY19335 + CS		1.13	Strong	
MRSA (USA 200)				
W7749	(ST36-SCC*mec*II)	0.29	Weak	Downregulation
MW7749	Δ*agr*::*tetM*	0.58	Moderate	($P<0.01$)
MSSA				
HC296	*mecA*	0.14	Non-producer	Downregulation
MHC296	Δ*agr*::*tetM*	0.36	Weak	($P<0.001$)
MRSA (USA 100)				
NY19339	(ST5-SCC*mec*II)	0.21	Non-producer	Downregulation
MNY19339	Δ*agr*::*tetM*	0.61	Moderate	($P<0.001$)
CMNY19339	Δ*agr*::*tetM*, p*bla-rnaIII*	0.3	Weak	
NY19339 + CS		0.73	Moderate	

*BU, biofilm unit. For statistical analysis the test of hypotheses on two population means was used to compare the BU values of the WT and the *agr*-null constructs. The null hypothesis (H_0: $\mu=\mu_0$) was rejected for $P<0.05$.

†CS, Conditioned supernatant: bacterial supernatant containing heterologous AIP was added to the bacterial inoculum at a final concentration of 10 % to impair *agr* function.

Gene Images Alkphos Direct Labelling and Detection System with CDP STAR (GE Healthcare).

Biofilm. Biofilm assays were performed in 96-well Nunclon microtitre inert polystyrene plates (Nunc), using trypticase soy broth (TSB) (Difco) supplemented with 1 % (w/v) glucose (TSB-1 % Glc) as described previously (Amaral *et al.*, 2005). Briefly, bacteria were grown in TSB-1 % Glc, in a shaker (250 r.p.m.) at 37 °C for 18 h. Cultures were diluted 1 : 100 in TSB-1 % Glc and 200 µl was inoculated into each well. The microtitre plate was incubated at 37 °C for 20 h. Supernatants were removed from each well and biofilms were gently washed twice with 0.85 % NaCl, then dried and fixed at 65 °C for 1 h. Finally, the plates were stained with crystal violet (Gram-stain), gently washed twice, and the absorbance was read in a microplate reader at 570 nm ($A_{biofilm}$). In parallel, the A_{570} of 1/100 cultures in TSB-1 % Glc (incubated in static conditions at 37 °C for 20 h) was also determined (A_{growth}). The biofilm unit (BU) was calculated using the following formula: $BU = A_{biofilm}/A_{growth}$. Based on the results obtained for the negative control *Streptococcus pyogenes* 75194 (BU=0.115), the isolates were classified as non-producers (BU$\leqslant$0.230), weak producers (BU>0.230 and $\leqslant$0.460), moderate producers (BU>0.460 and $\leqslant$0.920) and strong producers (BU>0.920). Because the development of biofilm is subject to phase variation, tests were repeated eight times. At least two independent experiments were carried out for each test. The mean BU value was used for the statistical calculation.

In addition, to confirm the differences between biofilm phenotypes, as determined by BU values, confocal laser scanning microscopy (CLSM) was employed to record and contrast structural images of the biofilms formed for representatives of clinical isolates displaying different biofilm phenotypes and also for the *agr* mutants derived from isolates HC474 and NY19335. The biofilm assays were performed as above, but after being fixed, all cells within the biofilm were stained with 25 nM SYTO 9 DNA-intercalating stain (Invitrogen) for 15 min in the dark. The stain was gently removed and biofilm was visualized using a Zeiss LSM510 metalaser scanning confocal microscope. The microscope was inverted and configured with one laser (argon 458 nm/477 nm/488 nm/514 nm). Images were captured at random with a Plan-Neofluar 40 × /0.6 Korr objective. Filters were set to a band pass of 500–530 nm.

Statistical calculations. Student's *t*-test (unpaired data) was used to compare the mean BU values. The null hypothesis (H_0: $\mu=\mu_0$) was rejected at level α=0.05 (Dunn, 1964).

***agr* interference using conditioned supernatant.** It was shown that the effect of a conditioned supernatant (CS) and purified heterologous Agr autoinducing peptide (AIP) in inhibiting *agr* activation was identical for all *agr* types tested (Ji *et al.*, 1997; Jarraud *et al.*, 2000; Novick *et al.*, 2000). Although we carried out RNAIII trans-complementation studies to check our genetic analysis, we also performed the same simple test as an additional control of the experimental system. Thus, 0.1 vol. CS (obtained from a stationary phase culture sterilized by filtration) was added to the bacterial inoculum (previously diluted 1 : 100) in TSB-1 % Glc. Then 200 µl of this mixture was placed in each well and assayed for biofilm formation using microtitre plates, as described above. CS type I (prepared using isolate BMB9393) was used to impair the Agr system of the *agr* type II isolates USA 100 and MSSA cultures. CS type II (prepared from isolate USA 100) was used to impair *agr* of the BEC isolates BMB9393 and GV69. In parallel, the experiment was performed exactly as described above but using filtered culture supernatant containing homologous AIP, for controlling possible effects of other bacterial products that might interfere in the system.

Haemolytic activity on blood agar plates. The δ-haemolysin (Hld), encoded by the *hld* gene, is encoded within the *rnaIII* region (Janzon & Arvidson, 1990). Thus, preliminary, BEC isolates (ST239-SCC*mec*IIIA) were screened for haemolytic activity on sheep red blood agar plates. A fresh culture of strain RN4220 (grown at 37 °C for 18 h on trypticase soy agar; TSA) was stripped in the central region of the plate. Then test isolates (also grown on TSA) were inoculated perpendicular to RN4220, in each half of the plate. The plate was incubated at 37 °C for 18 h and examined for a haemolytic zone surrounding the bacterial growth. Because β- and δ-haemolysins lyse sheep red blood cells synergistically, the production of the δ-haemolysin frequently results in an arrow-tip-like zone where δ-haemolysin overlaps with β-haemolysin produced by RN4220 (Adhikari *et al.*, 2007). Representatives of BEC isolates that produced very weak or undetectable haemolytic activity on blood plates were selected for Northern-blotting experiments with the RNAIII-specific probe.

DNA sequencing. Purification of PCR products was carried out using the QIAquick PCR Purification kit (Qiagen), as recommended by the manufacturer. DNA sequencing was carried out by primer walking using a MegaBACE 1000 automatic sequencer (GE Healthcare). Sequences were multiply aligned with the most similar sequences available at GenBank; CLUSTAL W was used to align the sequences.

RESULTS

Bacterial constructs

The presence of RNAIII transcript in the WT isolates (seven MRSA and five MSSA) was confirmed by RT-PCR and Northern blotting. The *agr* locus was entirely deleted in these isolates by allele replacement. MRSA *agr*-null constructs (Δ*agr*::*tetM*) were obtained from representatives of USA 100 (five isolates), USA 200 (one isolate) and USA 400 (one isolate) lineages. *agr* elimination was confirmed by resistance to tetracycline, absence of PCR amplification using *rnaIII*-specific primers, and absence of chemiluminescence signals after dot hybridization with the *rnaIII* probe. Among these 12 *agr*-null constructs, four were amenable to complementation experiments by transferring pRN6848 (pRN5548::*rnaIII-cat*) using phage transduction. The *rnaIII*-complemented mutants were confirmed by concomitant tetracycline and chloramphenicol resistance, PCR amplification of the *rnaIII*-specific fragment and dot-blot hybridization using the *rnaIII* probe. PFGE experiments confirmed the relatedness of each set of WT, *agr*-null and -complemented mutants (data not shown). In addition, trans-complemented mutants were able to transcribe RNAIII as showed by the Northern-blotting experiments. All the isolates and constructs used in this study are listed in Table 1.

Variability in the biofilm phenotype among *S. aureus* clinical isolates

We have previously reported that variants within the same MRSA clone (ST239) can display different biofilm phenotypes (Amaral *et al.* 2005). Thus, we decided to evaluate biofilm formation/accumulation in the clinical

isolates of *S. aureus*, in order to select representatives of each different phenotype for the experiments involving genetic manipulations.

Previous studies demonstrated that the *in vitro*, under static or flow conditions, and *in vivo* analysis of biofilm formation correlated quite well (Toledo-Arana *et al.* 2005; Beenken *et al.* 2004; O'Neill *et al.*, 2008). Thus, because multiple isolates were tested here and based on the observation above, the semiquantitative method chosen for measuring biofilm formation was primarily carried out on polystyrene microtitre plates and validated by CLSM experiments. The isolates were classified as strong, moderate, weak or non-biofilm producers based on bacterial cell density as determined by spectrophotometry.

Using these criteria, three of the clinical isolates selected were classified as biofilm non-producers (MSSA: HC410, HC296; USA 100: NY19339) and nine as biofilm producers. Among the producers, four were classified as strong (MSSA: HC474, HC642; USA 100: NY17859; USA 400: WB81), with BU values varying from 1.01 to 2.81, and five as weak producers (USA 100: NY17896, NY22735, NY19335; MSSA: HC569; USA 200: W7749). The BEC isolates BMB9393 and GV69 included in this study were classified as strong and moderate biofilm producers, respectively (Table 1). Fig. 1 shows the three-dimensional topography of *S. aureus* biofilms and the topometry values obtained from CLSM experiments for representatives of different biofilm phenotypes. The strong biofilm producer BMB9393 (BU=2.78) formed a dense lawn 47.1 μm thick (Fig. 1a), the moderate biofilm producer GV69 (BU=0.90) displayed a more irregular film 26.6 μm thick (Fig. 1b) and the weak producer NY22735 (BU=0.38) developed a biofilm 18.4 μm thick (Fig. 1c). These data confirmed our previous findings (Amaral *et al.*, 2005) that the ability of *S. aureus* clinical isolates to accumulate glucose-induced biofilm on polystyrene surface can vary significantly.

Effect of *agr* on biofilm development

The fact that most previous studies addressing the effect of *agr* on *S. aureus* biofilm were conducted using a small number of well-known laboratory mutants motivated us to examine the impact of *agr* elimination on contemporary clinical isolates of MSSA/MRSA displaying different biofilm phenotypes. Given that ST239 isolates are resistant to most antimicrobial drugs, *agr* mutation Δ*agr*::*tetM* was carried out only in MRSA lineages that displayed tetracycline susceptibility (Tc^s).

The deletion of *agr* in all four Tc^s isolates classified as strong biofilm producers exerted a decrease in the biofilm density, varying from about two- to eightfold reduction when compared with WT ($P<0.01$ to $P<0.001$; Table 1). We succeeded in trans-complementing RNAIII in one of these *agr*-null constructs, the MHC474 mutant. The biofilm defect shown by MHC474 (BU=0.36) was reverted (BU=0.95) by the introduction of the complementing plasmid pRN6848 into this construct (Fig. 2a, panels 1–3 and Fig. 2b, wells 1–3). Fig. 2(c) shows the result of RNAIII transcription for the set of WT, *agr*-null and *rnaIII*-complemented constructs. These data suggest that RNAIII upregulates the strong biofilm accumulation developed by these isolates on the static surface of polystyrene plates.

Consecutively, we examined the effect of *agr* elimination on the biofilm phenotype of eight weak or non-producer isolates (Table 1). For three of these isolates, the elimination of *agr* caused no significant alteration in the biofilm phenotype when compared with WT (USA 100: NY17896 and NY22735, weak producers; MSSA: HC410, biofilm non-producer). These results suggested that the inability of some *S. aureus* isolates to form or accumulate biofilm on polystyrene surface was not influenced by *agr*. Nevertheless, the Δ*agr*::*tetM* mutation in three isolates displaying a weak biofilm phenotype (MSSA: HC569; USA

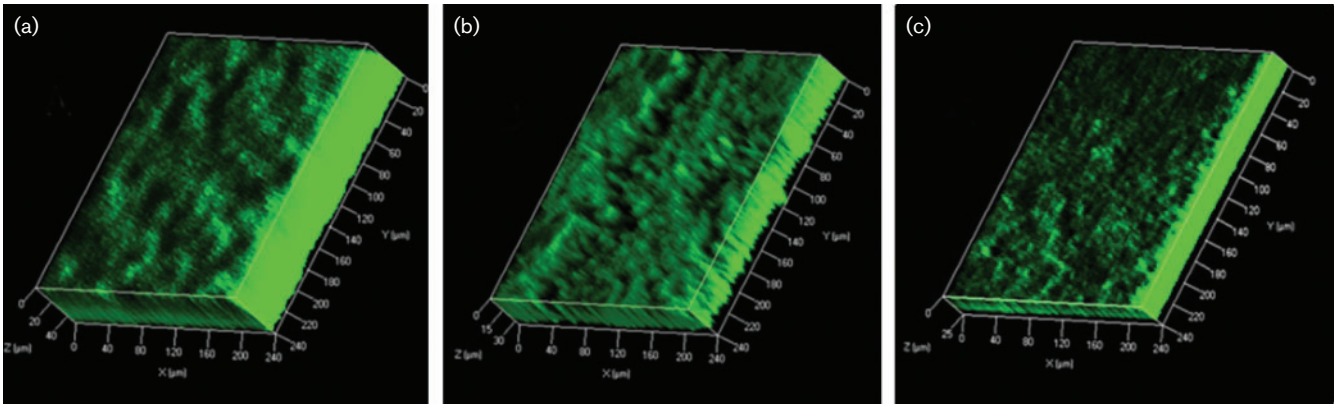

Fig. 1. Topographical images obtained by CLSM of different biofilm phenotypes expressed by *S. aureus* isolates, representative of two separate experiments. (a) Strong biofilm phenotype formed by isolate BMB9393 (47.1 μm thick). (b) Moderate biofilm phenotype formed by the isolate GV69 (26.6 μm thick). (c) Weak biofilm phenotype formed by the isolate NY22735 (18.4 μm thick).

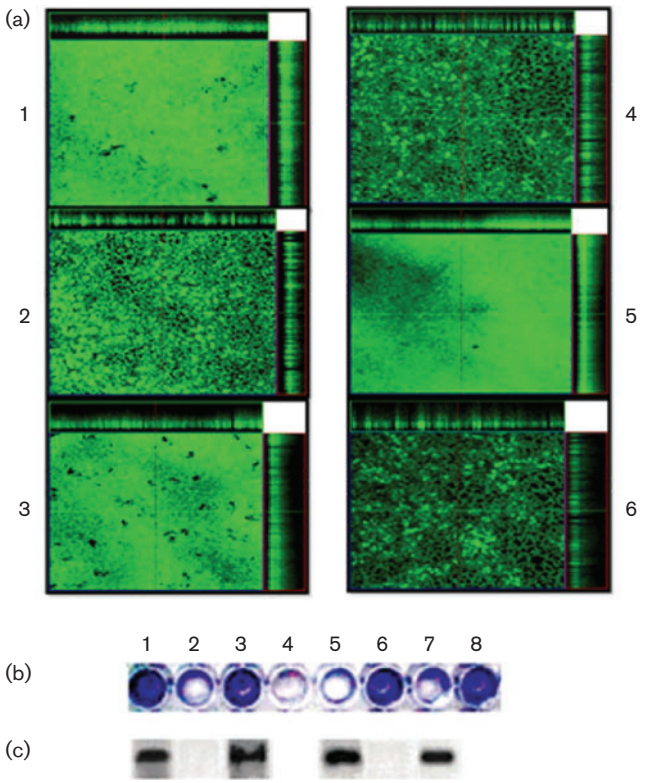

Fig. 2. Divergent effect of RNAIII on *S. aureus* biofilms. (a) CLSM image reconstructions representative of three independent experiments. Biofilms were formed on inert polystyrene surface and treated with SYTO 9. The vertical (red) line indicates the location of the *Y* plane from which the cross-section was taken. The horizontal (green) line indicates the location of the *X* plane from which the cross-section was taken. The blue lines indicate the slice of the biofilm from which the *XY* image was taken. Panels: 1, HC474 (WT, MSSA); 2, MHC474 (Δ*agr*::*tetM*); 3, CMHC474 (Δ*agr*::*tetM*, p*bla-rnaIII*); 4, NY19335 (WT, ST5-SCC*mec*II); 5, MNY19335 (Δ*agr*::*tetM*); 6, CMNY19335 (Δ*agr*::*tetM*, p*bla-rnaIII*). (b) Biofilm formed on microtitre plates and stained with crystal violet. Wells: 1, HC474 (WT, MSSA); 2, MHC474 (Δ*agr*::*tetM*); 3, CMHC474 (Δ*agr*::*tetM*, p*bla-rnaIII*); 4, HC474 treated with 0.1 vol. conditioned supernatant (CS); 5, NY19335 (WT, ST5-SCC*mec*II); 6, MNY19335 (Δ*agr*::*tetM*); 7, CMNY19335 (Δ*agr*::*tetM*, p*bla-rnaIII*); 8, NY19335 treated with 0.1 vol. CS. (c) Northern-blot experiments using specific RNAIII probe. Lines: 1, HC474 (WT, MSSA); 2, MHC474 (Δ*agr*::*tetM*); 3, CMHC474 (Δ*agr*::*tetM*, p*bla-rnaIII*); 4, empty space; 5, NY19335 (WT, ST5-SCC*mec*II); 6, MNY19335 (Δ*agr*::*tetM*); 7, CMNY19335 (Δ*agr*::*tetM*; p*bla-rnaIII*); 8, empty space.

100: NY19335; USA 200: W7749) had a positive impact on biofilm accumulation, corresponding to about two- to fourfold increase in the BU values ($P<0.01$ to $P<0.001$; Table 1). We were able to introduce pRN5548::*rnaIII-cat* into two of these *agr*-null mutants. As a result, the strong biofilm phenotype achieved by the construct MNY19335 (BU=1.28) dropped to the level of the isogenic WT (BU=0.33) after the RNAIII trans-complementation (BU=0.3; Fig. 2a, panels 4–6 and Fig. 2b, wells 5–7). Similarly, the trans-complementation of the MSSA mutant MHC569 (BU=0.69) caused a reduction in biofilm density (BU=0.42) closer to the levels of the corresponding WT (BU=0.35; Table 1). Thus, these data show that *agr* downregulated biofilm accumulation in three isolates displaying a weak biofilm phenotype. In addition, the removal of *agr* induced biofilm formation in two non-producer isolates (MSSA: HC296; USA 100: NY19339). The impairment of biofilm formation caused by the presence of the RNAIII transcript was confirmed by trans-complementation. The introduction of pRN5548::*rnaIII-cat* into MNY19339 restricted biofilm formation to the level of the WT (Table 1). These results indicate that RNAIII can block the development of biofilm in some isolates of *S. aureus*.

It is important to mention that the biofilm development of isolates within the same lineage (quite similar PFGE pattern, the same ST5 and SCC*mec*II) was found to be either up- or downregulated by *agr*, or even unaffected. For example, the biofilm accumulation of the USA 100-related isolate NY17859 (strong producer) was upregulated by *agr*, whereas RNAIII downregulated biofilm accumulation of the USA 100 isolate NY19335 (weak producer) and inhibited biofilm formation of the isolate NY19339 (normally a non-producer). However, RNAIII did not affect the biofilm phenotype of the USA 100-related isolates NY17896 and NY22735, both weak producers (Table 1).

Agr interference using conditioned supernatant

Previous studies have provided strong evidence that the only *agr*-activating component in the supernatant of *S. aureus* isolates that are Agr^+ is related to the presence of AIP (Novick *et al.* 2000; Shaw *et al.* 2007). Thus, beside RNAIII complementation, the *agr* interference using conditioned medium (Ji *et al.*, 1997) could provide a simple and straightforward experiment to quickly recheck the effect of *agr* in *S. aureus* biofilm formation on microtitre plates. In fact, the treatment with 0.1 vol. CS of four WT isolates displaying strong (HC474, BU=1.12) or weak (HC569, BU=0.35 and NY19335, BU=0.33) biofilm formation, and a biofilm non-producer (NY19339, BU=0.21) consistently reproduced the effect of *agr* deletion on biofilm development. Thus, the BU values obtained after *agr* knockout and from CS experiments were HC474, 0.36 and 0.39; HC569, 0.69 and 0.62; NY19335, 1.28 and 1.13; and NY19339, 0.61 and 0.73. Fig. 2(b) shows the data of the *agr*-interference experiments for two of these isolates (Fig. 2b, well 2, MHC474 and well 4, HC474+CS; well 6, MNY19335; and well 8, NY19335+CS). Taken together, the results from *agr*-null mutants, RNAIII trans-complementation and also from 0.1 vol. CS were all consistent with the participation of RNAIII regulation on biofilm formation/accumulation of *S. aureus* isolates.

Biofilm development of BEC isolates GV69 and BMB9393

We have found that some BEC isolates can display a natural *agr* impairment, as first revealed by screening of haemolytic activity on blood agar and confirmed by Northern-blot hybridizations (Fig. 3a, b). Thus, we selected the isolate GV69 (*agr* attenuated) to compare its ability to form/accumulate biofilm with that of BMB9393, which had stronger RNAIII signals in the Northern blotts (Fig. 4a, upper panel). Our results showed that BMB9393 developed a strong biofilm phenotype (BU=2.78; Fig. 3c, well 1), threefold more dense than that of GV69 isolate (BU=0.90; Fig. 3c, well 2). However, despite the fact that RNAIII expression was very low in GV69 compared with BMB9393 (Fig. 4), the biofilm developed by GV69 could still accumulate on a polystyrene surface (Fig. 1b). Because BEC isolates are tetracycline resistant (Tc^r), we tested the effect of 0.1 vol. CS in the biofilm developed by these two isolates. The CS experiment with the isolate GV69 resulted in further decrease in the BU value to 0.48 (Fig. 3c, wells 3 and 4). Similarly, the biofilm formed by BMB9393 significantly reduced to BU=0.78 after CS treatment (Fig. 3c, wells 5 and 6). Analogous to what was observed for the *agr* knockouts derived from strong biofilm producers, these data corroborate the evidence that the stronger biofilm accumulation by some isolates of *S. aureus* seems to be upregulated by *agr*.

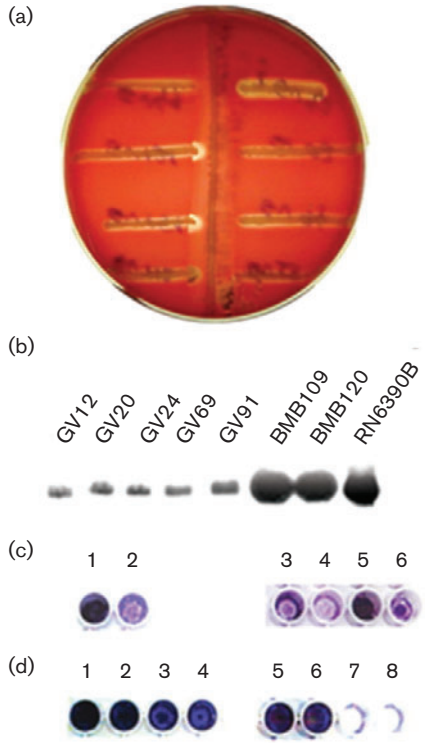

Fig. 3. (a) Haemolytic activity on sheep blood agar. Vertical strip, β-haemolysin-producing strain RN4220; horizontal strips, BEC isolates. Note that *agr* naturally attenuated isolates displayed absence of an arrow-tip-like zone on the intersection with the RN4220 strip and weak haemolytic activity. (b) Northern-blot experiments showing lower RNAIII expression for BEC isolates GV12, GV20, GV24, GV69 and GV91. *S. aureus* isolates BMB109, BMB120 and RN6390B, displaying strong RNAIII signals, were used as controls. (c) Biofilm expression of the BEC isolates (ST239-SCC*mec*IIIA). Wells 1, BMB93932 and 2, *agr* naturally attenuated GV69. Wells 3, untreated BMB9393 and 4, BMB9393 treated with 0.1 vol. CS. Wells 5, untreated GV69 and 6, GV69 treated with 0.1 vol. CS. (d) Investigation of the importance of carbohydrates and of protein(s) in biofilm structural composition of BEC strains. Wells 1 and 2, untreated BMB9393; 3 and 4, BMB9393 after treatment with sodium metaperiodate; 5 and 6, untreated BMB9393; 7 and 8, BMB9393 after treatment with proteinase K.

Previous studies have suggested that *S. aureus* biofilm under glucose induction is composed mainly of protein (O'Neill *et al.*, 2007). In order to validate this observation, we treated the pre-formed biofilm of isolate BMB9393 with 100 µl 10 mM sodium metaperiodate (to oxidize carbohydrates) or proteinase K (1 mg ml^{-1}) for 2 h at 37 °C, as suggested by Chaignon *et al.* (2007). We also found metaperiodate treatment had only a moderate effect on the strong biofilm accumulated by this isolate (Fig. 3d, wells 1–4). However, proteinase K removed virtually all biofilm formed by BMB9393 (Fig. 3d, wells 5–8), confirming the importance of protein component(s) in this process.

agr and *sarA* sequencing of BEC isolates

In view of the difference in RNAIII expression between the BEC isolates (Fig. 4a), the whole *agr* locus of isolate GV69 was sequenced and compared with that of BMB9393 and RN6390B (also an *agr* type I isolate). Sequencing revealed that the *agr*-attenuated isolate GV69 had a unique point mutation in the sequence encoding the 3′ end of RNAII, corresponding to a substitution of guanine for thymine (dbSNP build130: ss# 8610943). Despite the fact that this mutation was not found in the RN6390B sequence, the same alteration was detected in the isolate BMB9393 (Fig. 4b), which displayed a strong RNAIII signal. Thus, the alteration in RNAII could not be implicated in the *agr* attenuation detected in GV69 and might be a common characteristic of the lineage ST239. We can infer that the defect would be located upstream of *agr*.

Given that SarA is a positive regulator of *agr*, we speculated that the *agr* attenuation in GV69 could be due to a mutation in *sarA*. We carried out *sarA* Northern blotting and verified, as expected, that *sarA* was also attenuated in this BEC isolate but not in BMB9393 (Fig. 4a, middle panel). The lower expression of *sarA* mRNA may therefore explain the *agr* attenuation detected. Next, we decided to sequence the regions encompassing all *sar* promoters (P1, P2 and P3). Alignments of the sequences from isolate GV69 (GenBank accession number EU301433) with the equivalent segments from *S. aureus sar* sequences at GenBank did not show any variation in these regions (data not shown).

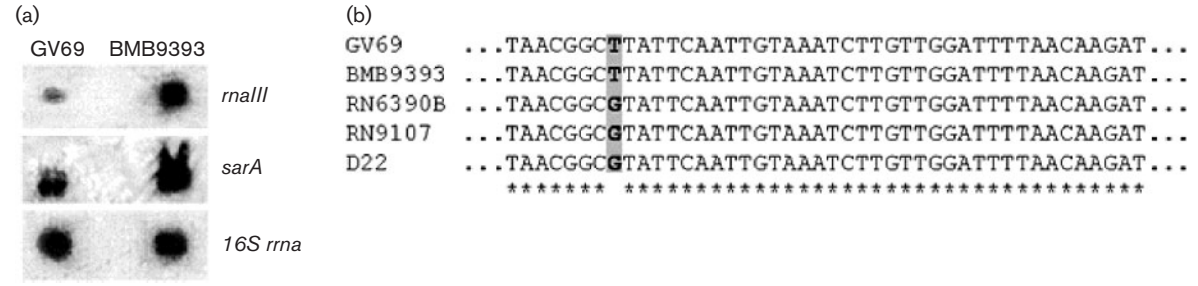

Fig. 4. (a) Northern-blot experiments with BEC isolates BMB9393 and GV69 (ST239-SCC*mec*IIIA) using *rnaIII*, *sarA* and *16S-rrna* specific probes. (b) Multiple alignment of the 3′ region of the *agr* RNAII showing a point mutation in the BEC strain GV69 and BMB9393 (*agr* type I), RN6390B (*agr* type I), RN9107 and D22 (GenBank accessed sequences of the *S. aureus* isolates RN9107 and D22, both *agr* type I). Grey shading shows the point mutation, the replacement of a guanine by thymine. Asterisks represent the consensus sequence.

These data indicated that the responsible defect for *agr*/*sarA* attenuation is likely to be upstream of *sar*.

DISCUSSION

The accessory gene regulator *agr* is one of the main global virulence regulators in *S. aureus*. However, the effective role played by *agr* in biofilms has not been fully elucidated. Knowing how *agr* can affect *S. aureus* biofilms is critical to our understanding of the dynamics involved in the formation, accumulation and detachment of these bacterial films. To further investigate whether the *agr* RNAIII has a function in biofilm formation/accumulation in clinical isolates of *S. aureus*, we analysed *agr*-null mutants derived from contemporary isolates of both MSSA and MRSA. The experiments were designed to have the mutation Δ*agr*::*tetM* transferred into isolates representative of different biofilm phenotypes. Essentially, the results obtained from the majority of *agr* knockouts indicated that *agr* could either promote or inhibit glucose-induced biofilm on polystyrene inert surfaces, under static conditions. Only with a few isolates did *agr* deletion not affect biofilm formation or accumulation (3/12; 25 %). Thus, we found that *agr* modulation could diverge from up- to down-regulation in *S. aureus* isolates displaying different biofilm phenotypes. Yarwood *et al.* (2004) showed that under some conditions disruption of *agr* expression could have no detectable influence on *S. aureus* biofilms, while under others it could either inhibit or enhance biofilm formation.

Here we showed that the phenotype of four strong biofilm producers changed to weak (three isolates) and to moderate (one isolate) when *agr* was removed. We could restore the biofilm defect by reintroducing *rnaIII* into one of these knockouts and reproduce the biofilm phenotype of the *agr*-null mutant by interfering with the *agr* function using conditioned supernatant (0.1 vol. CS). Therefore, it is reasonable to conclude that *agr* exerted a positive regulation on biofilm accumulation in these strong biofilm-forming MSSA/MRSA isolates.

Conversely, *agr* downregulated the biofilm expression in three of five isolates classified as weak biofilm producers. We also found that two isolates classified as non-producers had the ability to form biofilm after *agr* removal (down-regulation). RNAIII-complementation experiments for three of these isolates regenerated the biofilm phenotype to the levels of the corresponding WT. Here again, the results of the interfering experiments with CS reproduced the biofilm impact observed in the *agr* knockouts. These data are in agreement with previous studies showing *agr* had a negative impact on biofilm development by *S. aureus* (Vuong *et al.*, 2000). However, a small impact of *agr* on biofilm formation was suggested by others, using different substrates for biofilm development. In contrast, these researchers found that *sarA* deletion caused a significant decrease in the *S. aureus* biofilm (Beenken *et al.*, 2003; Valle *et al.*, 2003). In fact, there is growing evidence for a role of *sarA* in the upregulation of biofilm formation by *S. aureus* (O'Neill *et al.*, 2007).

The contradictory findings for the role of *agr* on biofilm formation have been attributed to the fact that many studies have used strain NCTC 8325-4 or derivatives, which also have mutations in the *sigB* operon and in *agrA* (Kullik *et al.*, 1998; Adhikari *et al.*, 2007). In addition, biofilm formation by 8325-4 varies according to the laboratory source of this strain (Valle *et al.*, 2003). To get around this, O'Neill *et al.* (2007) also used a number of clinical isolates and found that *agr* mutations significantly increased biofilm (more than twofold) in 5 of 21 (23 %) clinical isolates but had no significant impact on biofilm formation in the remaining 16.

Biofilm development seems to be a multifactorial process (Otto, 2008). It is well known that *agr* regulation can target different bacterial products, downregulating surface adhesins and upregulating secreted proteins (Novick *et al.*, 1993). As such, it seems likely that a variable composition of *S. aureus* biofilms would account for the observed differences in the biofilm phenotypes of the WT and also for the apparent discrepancy in the effect of *agr* on

biofilms. In fact, O'Neill *et al.* (2008) demonstrated that FnbpAB are involved in glucose-induced biofilm accumulation of *S. aureus*. Despite the fact that RNAIII downregulates Fnbps (Saravia-Otten *et al.*, 1997), the results of O'Neill and colleagues could not fully explain the role of *agr* on biofilms since *agr* deletion did not have a significant impact on 16 of 21 *agr*-null mutants tested (O'Neill *et al.*, 2007). Studies by Corrigan *et al.* (2007) showed that the expression of a homologue of the accumulation-associated protein (AAP) SasG by the laboratory strain SH1000 could also promote biofilm formation.

Moreover, not only *agr*-downregulated proteins but also the positively regulated ones have been implicated in *S. aureus* biofilm formation and accumulation. Recent studies by Johnson *et al.* (2008) showed that Eae and Emb secreted-adhesion proteins (both positively regulated by *agr*) played an important role in iron-regulated biofilm formation in *S. aureus*. Indeed, it was found that different environmental conditions other than iron could also affect the expression of *eae* and *emb* genes. The impairment of the *hla* gene significantly inhibited the adherence of strain NCTC 8325-4 to plastic surfaces under both static and flow conditions (Caiazza & O'Toole, 2003). Indeed, it was previously suggested that the elimination of the surfactant properties of the RNAIII-encoded δ-toxin was involved in the enhancement of the biofilm formed by the *agr*-transposon mutant *mut6* (Vuong *et al.* 2000).

More recently it was suggested that glucose-induced biofilm was mainly mediated by protein(s) (O'Neill *et al.*, 2007). Corroborating this hypothesis, significant impairment in biofilm formation was found in a knockout for sortase, which anchors LPXTG proteins, many of them negatively regulated by *agr* (O'Neill *et al.*, 2008). Our results also demonstrate that a protease could totally disrupt the strong biofilm formed by the isolate ST239. Thus, it is possible that differences in protease expression could also be implicated in the variability of biofilm formation/accumulation. Genes encoding extracellular proteases are generally repressed by SarA and positively regulated by RNAIII (Boles & Horswill, 2008). It was found that a *clpP* protease mutant formed more biofilm compared with WT cells (Frees *et al.*, 2004). In addition, experiments demonstrated that a double mutant *aur splABCDEF* (encoding the proteases aureolysin and Spl serine proteases, respectively) had minimal protease activity, enhanced biofilm formation and attenuated detachment phenotypes (Boles & Horswill, 2008). Yarwood *et al.* (2004) have already suggested that *agr* could influence biofilm dispersion. Taking our data together with the studies cited above, it can be concluded that the process of biofilm development in *S. aureus* is especially complex, of multifactorial nature and regulated by a coordinate network of regulatory systems including Agr, SarA and bacterial proteases.

Although it is difficult to directly transfer knowledge from experimental laboratory data to what occurs during clinical infections, the results presented here suggest that more detailed studies are needed to clearly evaluate the potential of *agr* inhibitors, such as RIP (Balaban *et al.*, 2007), as strategy for the development of *S. aureus* anti-biofilm agents. It would be of interest for future studies to include *S. aureus* clinical isolates displaying different biofilm phenotypes to test the effect of *agr*-inhibiting peptides using *in vivo* models, given that our *in vitro* studies showed *agr* knockout could enhance weak biofilms and allow biofilm formation of some non-producer isolates. Our findings that Agr can downregulate biofilm formation in some *S. aureus* isolates was supported by others (Vuong *et al.*, 2000; O'Neill *et al.*, 2007).

MRSA isolates of ST239 are widespread in Brazil and in many other countries (Teixeira *et al.*, 1995). One of the most striking properties of this lineage is its superior ability to adhere to and invade human airway cells and to form an enhanced biofilm on polystyrene inert surfaces (Amaral *et al.*, 2005). Some ST239 variants are *agr*/*sarA* naturally attenuated. We showed that these attenuations were paralleled by more than twofold reduction of the ability to form glucose-induced biofilm. RNAIII attenuation is not a rare phenomenon in *S. aureus* clinical isolates (Papakyriacou *et al.*, 2000; Yarwood *et al.*, 2007). Although our results indicated that *sarA* attenuation affected RNAIII expression in the GV69 isolate, we could not detect any alteration in the sequence regions of *sar* promoters. Preliminary studies using transcriptional profiles indicated that the *sarA* repressor, *sarR*, was upregulated in the GV69 stationary phase but not in the BMB9393 (A. M. S. Figueiredo *et al.*, unpublished data). The occurrence of naturally *agr*-attenuated variants in *S. aureus* biofilms was recently reported by Yarwood *et al.* (2007). These studies revealed that *sarU* (a gene also involved in *agr* activation) was repressed in the non-haemolytic biofilm variant. All these data together implicate the Sar family of regulators as potential regulatory paths for the *agr* impairment of some MRSA clinical isolates. It is well recognized that *agr* and also *sarA*, as well as other gene regulators, coordinate the global expression of bacterial virulence factors (Novick *et al.*, 2000). Thus, it is logical to suppose that variations in *sarA*/*agr* expression among clinical isolates might represent an important mechanism of host adaptability and may contribute to the bacterial fitness in a specific epidemiological scenario.

In conclusion, *agr* elimination had considerable impact on glucose-induced biofilm formation/accumulation for the majority of clinical isolates tested. This effect could be reversed by trans-complementation of RNAIII into *agr* knockouts. The effect exerted by *agr* could be neutral, or up- or downregulation. Further insights into biofilm regulatory pathways involving *agr* would require the determination of the key molecule(s) involved in biofilm expression in *S. aureus*. It remains to be clarified if the differences observed in the biofilm phenotypes are due to a

multi-component structure of the *S. aureus* biofilms and/or the modulation of the biofilm-associated gene(s).

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