RESEARCH ARTICLE

agr-Genotyping and transcriptional analysis of biofilm-producing *Staphylococcus aureus*

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Abstract

We investigated the correlation between biofilm production and the accessory-gene-regulator (*agr*) in 29 strains isolated from catheter-associated infections compared to a control group (30 isolates). All strains were tested for their ability to produce biofilm in a static system, and their *agr* genotype was determined. ScaI-restriction fragment length polymorphism for *agr*-typing showed that strong biofilm-producing strains belong to *agr*-type II. We found two new *agr*-variants, and sequence analysis of the three PCR products revealed the insertion of IS*256* within the *agr*-locus. Biofilm production was assessed and correlated with *agr* functionality, with the expression of the *ica*-operon and of two transcriptional regulators, *sar*A and *rsb*U. Our data show that *agr*-II strains produce large amounts of biofilm, possess a defective *agr*-system show early transcription of *ica*A and are defective in haemolysin activity, *ica*R transcription, and in the expression of the σ^B activator *rsb*U. Strains with *agr*III are medium biofilm producers, have an inactive *agr*-system, but express *ica*AR and *rsb*U in the late- and postexponential growth phases. In *agr*I–IV- and -IA-variants, medium or weak biofilm production was found. In these strains, the *agr*-locus was fully functional, *rsb*U-*ica*R and *ica*A were found in the late- and/or postexponential phases. Biofilm production was not affected by *sar*A.

Introduction

Staphylococcus aureus is a major cause of both community- and hospital-acquired infections; it is permanent member of the human microbial flora, responsible for a myriad of infections ranging from subcutaneous abscesses or furuncles to scalded skin syndrome, sepsis necrotizing pneumonia, and toxic shock syndrome (TSS). Its pathogenesis is due to both toxin secretion and cell wall-associated protein production involved in adhesion and protection against host defenses. The increase of methicillin-resistance and the recent emergence of vancomycin-resistant *S. aureus* strains on the one hand, and the ability to form biofilm on indwelling medical devices on the other, make the infections associated to these microorganisms an important public health problem today (Lowy, 1998).

To cause so many human diseases and to colonize different ecological niches within the host, *S. aureus* has developed a quorum-sensing system that via cell-to-cell communication controls the regulation of a multitude of virulence factors. This system is based on a two-component module, known as the *agr*-locus (accessory-gene-regulator) that, in a cell density-dependent manner and by a secreted auto-inducing-peptide (AIP), allows a bacterial population to respond in concert when a critical cell-density is reached.

The *agr*-locus consists of the four genes *agr*BDCA, cotranscribed by a unique promoter, P_2, and a genetic region, transcribed by the P_3 promoter in the opposite direction, encoding the *agr* system effector molecule (RNAIII), which contains the *hld* gene responsible for δ-toxin production. The P_3 transcription activation determines the up-regulation of genes encoding for exoproteins and down-regulates the genes encoding cell-wall surface proteins. The P_2 transcript is required for transcriptional activation of the two *agr* promoters. AgrCA is a histidine-kinase two-component system in which AgrC is the signal receptor and AgrA is the response regulator. AgrD produces, orchestrated by AgrB, an auto-inducing peptide (AIP) with

a thiolactonic ring structure, which, in the early exponential phase, causes the immediate activation of the two promoters (Jarraud *et al.*, 2000, 2002; Novick, 2003). Although structurally conserved, *agr*B, D and C have diverged widely among staphylococci, giving rise to multiple specificity groups in which heterologous AIP-receptor interactions are inhibitory. Based on polymorphisms in the auto-inducing peptides, *S. aureus* can be classified as belonging to one of four allelic groups on the basis of AIP cross-inhibition of the *agr* response: these four AgrD auto-inducing peptides need different AgrB and AgrC protein-receptors to retain the specificity of receptor–ligand interaction (Balaban & Novick, 1995; Ji *et al.*, 1995, 1997; Davies *et al.*, 1998; Novick, 2000; Moore & Lindsay, 2001; Li *et al.,* 2002; Goerke *et al.*, 2003; Bronner *et al.*, 2004).

Many staphylococcal infections are associated with communicating cell groups, known as biofilms, in which cells are attached to abiotic or biotic surfaces and possess an altered growth and gene expression profile compared to planktonic (free-living) bacteria. Biofilm formation develops in two steps: (1) a primary attachment onto polymeric surfaces mediated, in part, by cell wall-associated adhesins including the microbial surface components recognizing adhesive matrix molecules ($MSCRAMM_S$); (2) cell–cell proliferation to form a multilayered biofilm mediated by production of extracellular factors such as the polysaccharide-intercellular-adhesin (PIA) (Mack *et al.*, 1996; Götz, 2002; Vuong & Otto, 2002).

In staphylococci, PIA is produced by the co-operation of proteins encoded by the *ica*-operon (inter-cellular-adhesin). The *agr*-locus may condition the *in vitro* biofilm behaviour and many *agr* regulated products, including α-toxin, surface-associated adhesins, δ-haemolysin and the AtlE autolysins. Vuong *et al.* (2000) reported that *agr*-null mutations contribute to enhancement of biofilm formation on polystyrene by an indirect mechanism, mediated by the surfactant properties of δ-toxin. The *agr*-locus normally down-regulates biofilm development and depends on the conditions in which the biofilm grows. It is clear that many regulatory mechanisms are involved in the biofilm phenotype of *S. aureus*, but their precise role is still poorly understood.

Recent reports indicated a relationship between virulence and antibiotic-resistance in *S. aureus*: it has been demonstrated that all glycopeptide-intermediate *S. aureus* strains (GISA) examined belonging to *agr*-group II were defective for *agr*-function as demonstrated by their inability to produce δ-haemolysin: in contrast, these strains were strong biofilm producers. These findings led to the hypothesis that GISA strains may exhibit diminished virulence and might have an enhanced ability to form a thick biofilm due to *agr*-locus inactivation (Sakoulas *et al.*, 2002, 2003).

Based on the above-mentioned information, the aim of the present study was: (1) to investigate the distribution of *agr*-alleles using a new ScaI-RFLP method; (2) to look at possible differences in the distribution of *agr*-types and biofilm production in strains isolated from CVCs and from other infection sites; (3) to analyze the expression of *ica*A, *ica*R, *agr*DCA, *hld, hla*, and the two regulators *sar*A and *rsb*U, in relation to biofilm production.

Materials and methods

Bacterial strains

Seventeen methicillin-resistant *S. aureus* (MRSA) and 12 methicillin-susceptible (MSSA) (numbers $1–29_{cvc}$) strains (multiple colonies of each strain from each sample were typed), isolated from central venous catheter infections (CVC), and 30 strains subdivided into 15 MRSA and 15 MSSA strains collected from various infections (numbers $1–30_{cg}$) used as control, were included. All strains were collected during an antibiotic resistance surveillance study from different Italian laboratories and were all epidemiologically unrelated, as demonstrated by pulsed-field gel electrophoresis (PFGE) (data not shown). All strains were grown on MSA (Mannitol–Salt–Agar, Oxoid, Basingstoke, UK) identified by coagulase test and by the Api-Staph System (Bio-Merieux, Marnes-la-Coquette, France) and maintained at $-80\,^{\circ}C$ until used for successive experiments. *Staphylococcus epidermidis* ATCC35984 was used as biofilm and *ica*-operon positive control.

Measurement of biofilm formation

The biofilm-forming ability of *S. aureus* was tested in a static system by a spectrophotometric quantitative assay. Each strain was grown in Tryptone Soy broth (Oxoid), with the addition of 0.25% glucose (TSBG). These assays were performed in microtitre plates as described previously (Christensen *et al.*, 1985; Ziebuhr *et al.*, 1999). Each reported value was the average of 12 measurements at 490 nm (± SD). Values of ≥ 0.12 were regarded as biofilm positive and values of > 0.4 were considered strong producers, 0.4–0.2 were medium producers and < 0.2 were considered weak producers. These absorbance values were a species-related modification of previously published values (Cafiso *et al.*, 2004).

agr-locus PCR and ScaI RFLP

Genomic DNA was isolated from each strain as previously described (Pitcher *et al.*, 1989). Amplification of the *agr*-locus was by PCR with CV15–16 primers (GenBank accession no. X52543) designed on the conserved regions of the four *agr* interference groups flanking the polymorphic regions that confer group specificity (Table 1).

Amplification mixes contained the following final concentrations: 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$,

Table 1. Primers, annealing temperatures and product length (bp) of the genes studied

Primer	Gene	Sequence	GenBank accession no.	RT T_m (°C)	PCR T_m (°C)	Product (bp)	Reference
CV15fwd	*agr*-locus	5′-CAG TGAGGAGAGTGGTGTAAAATT-3′	X52543	–	58	2583	This study
CV16rew		5′-AAATGGGCAATGAGTCTGTGAG-3′					
CV25fwd	*rna*III	5′-CTGAGTCCAAGGAAACTAACTCTAC-3′	X52543	60	56	235	This study
CV55rew		5′-ATCTTGTGCCATTGAAATCA-3′					
CV27fwd	*agr*D	5-CATTCCTGTGCGACTTATTAAACG-3′	X52543	54	56	307	This study
CV28rew		5-CGTGTAATTGTGTAAATTCTTTTGG-3′					
CV61fwd	*agr*C	5-GACTTCGCAGATTATTCTATACTGTG-3′	U85096	54	55	279	This study
CV62rew		5-CACAAATAAACTCGGATGAAGC-3′					
CV59fwd	*agr*A	5-TTAACAACTAGCCATAAGGATGTG-3′	U85096	60	60	310	This study
CV60rew		5-CGTTACGAGTCACAGTGAACTTAC-3′					
CV36fwd	*sar*A	5-CAATGGTCACTTATGCTGACAAAT-3′	U46541	56	50	379	This study
CV37rew		5-GGTGAAGTTTGATAGATGATACATTC-3′					
CV40fwd	*rsb*U	5-GATAAAGAGATAGACTTAGCTTCT-3′	Y07645	60	50	460	This study
CV41rew		5-ACGTTAGAGGTAGAGTGTTAGGAATCA-3′					
CV32fwd	*ica*R	5-AGTAGCGAATACACTTCATCTTTGA-3′	AF086783	60	50	499	This study
CV33rew		5-GTTGTACCGTCATACCCCTTCTCTG-3′					
CV30fwd	*ica*A	5- CATTGAACAAGAAGCCTGACA-3′	AF086783	54	50	301	This study
CV31Brew		5- ATATGATTATGTAATGTGCTTGGATG-3′					
CV23fwd	*hla*	5-AGGTTCCATATTGATGAATCCTG-3′	X01645.1	60	60	737	This study
CV24Brew		5-CAGACTTCGCTACAGTTATTACTATGG-3′					

150 mM KCl and 0.1% Triton X-100, 1 U of Taq-polymerase (DyNAzyme^{TM}II DNA polymerase, Finnzymes, Oy, Espoo, Finland), 200 μM each deoxynucleoside triphosphate and 1 μL of genomic DNA. PCR conditions comprised an initial 5 min denaturation step at 94 °C, followed by 30 cycles of 1 min at 93 °C, 30 s at 58 °C, 2 min at 68 °C, with a final annealing step of 1 min at 58 °C and extension for 10 min at 68 °C. After PCR, the amplicons were digested with ScaI restriction enzyme (New England Biolabs, Beverly, MA). This enzyme cuts the I-II-III agr-sequences at different sites, whereas there were no restriction sites in the agr-IV genotype. Amplicon digestion was carried out in a reaction mixture containing 8 U of ScaI, 1X ScaI buffer and incubated at 37 °C for 18 h. The restriction products were separated on 1% agarose gel and stained with ethidium bromide (0.5 μg mL^{-1}). The predicted restriction fragment sizes are shown in Fig. 1.

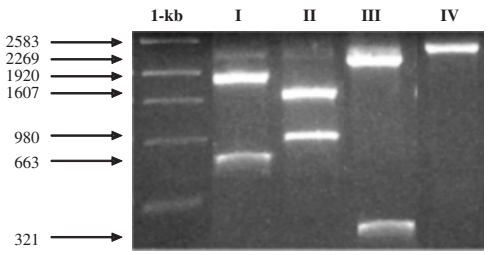

Fig. 1. Predicted restriction fragments of the four agr alleles after ScaI digestion.

PCR of other virulence genes

Amplification of *ica*R, *ica*A, *agr*D, *agr*C, *agr*A, *hld*, *hla*, *sar*A, and *rsb*U genes was performed using primers designed on the deposited gene sequences as shown in Table 1. Amplification mixes contained the following final concentrations: 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 150 mM KCl and 0.1% Triton X-100, 1 U of Taq-polymerase (DyNAzyme^{TM}II DNA polymerase, Finnzymes, Oy), 200 μM each deoxynucleoside triphosphate and 1 μL of genomic DNA. PCR conditions comprised an initial 5 min denaturation step at 94 °C, followed by 30 cycles of 1 min at 93 °C, 30 s at annealing temperature (Table 1), 1 min at 68 °C, with a final annealing step of 1 min at 68 °C and extension for 10 min at 68 °C. PCR products were separated on 1% agarose gel and stained with ethidium bromide (0.5 μg mL^{-1}).

DNA sequence analysis

The nucleotide sequences of PCR products were obtained by previously published methods (Sanger *et al.*, 1977). Sequencing was performed on a Li-Cor automated sequencing system (Li-Cor Biotechnology, Lincoln, NE) with infrared dye-labeled sequencing primers. The sequencing reactions were performed with a TermoSequenase fluorescent-labeled primer cycle sequencing kit (Amersham Bioscience, Chalfont St Giles, UK), used according to the manufacturer's instructions. The *agr*-locus PCR products were purified using Qiagen columns according to the manufacturer's instructions (M-Medical s.r.l., Genenco).

Study of expression

RNA isolation

Total bacterial RNA was extracted at different growth phases: in the mid-exponential ($OD_{600\,nm} = 0.2$ at 2.5 h after inoculation), in the late-exponential ($OD_{600\,nm} = 0.6$ at 5 h after inoculation), and the postexponential ($OD_{600\,nm} = 1.1$ at 7 h after inoculation). An aliquot of an overnight culture was diluted 1 : 50 in 50 mL TSBG and incubated at 37 °C. The cultures were then harvested and centrifuged at 4000 ***g*** for 30 min and resuspended in 200 μL of diethylpyrocarbonate (DEPC)-treated H_2O, 100 μL lysostaphine (50 μg mL^{-1}), 100 μL lysozyme (100 μg mL^{-1}). After incubation at 37 °C for 30 min, 1 mL of Trizol-reagent (GibcoBRL, Paisley, UK) was added and incubation continued for a further 5 min. Following incubation, 200 μL of chloroform was added, mixed by agitation; the mixture was incubated for 5 min, and centrifuged at 10 000 ***g*** for 15 min. After centrifugation, 1 mL of cold-isopropanol was added and the mix was incubated at room temperature for 30 min, this was followed by centrifugation at 12 000 ***g*** for 15 min, resuspension of the pellet in 50 μL of DEPC–H_2O and storage at −20 °C. Genomic DNA was removed by treatment with RNase-free-DNaseI (Ambion, Austin, TX) and the residual RNA concentration was determined spectrophotometrically. Each extracted RNA sample was also used as a template in the PCR assay to confirm the absence of DNA contamination.

RT-PCR

Nine CVC isolates and nine control strains randomly selected from the two study groups, belonging to the four *agr*-genotypes including the two variants (IA and IB), were cultured in the presence of glucose at different cellular densities and the expression of *ica*R, *ica*A, *agr*D, *agr*C, *agr*A, *hld*, *hla*, *sar*A, and *rsb*U was evaluated by RT-PCR. These assays were performed with a two-step procedure in which 200 ng of RNA was added to a mix comprising 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol pH 8.3, 200 μM of each deoxynucleoside triphosphate, 0.2 μM of each primers and 200 U of M-Mu LV-reverse-transcriptase (BioLabs). cDNA synthesis was performed for 10 min at the annealing temperature of the primers followed by incubation at 37 °C for 60 min. For PCR, 5 μL of the RT mixture was used as a template and PCR conditions comprised an initial 5 min denaturation step at 94 °C, followed by 30 cycles of 1 min at 93 °C, 30 s at the specific annealing temperature of the primers, 1 min at 68 °C, with a final annealing step of 1 min at the annealing temperature and extension for 10 min at 68 °C. All sequence primer and annealing temperatures, both in RT and PCR, are listed in Table 1. For each RT-PCR, an internal positive control, the *pta* gene RT, was used as positive RNA quality control and to verify the reliability of the results. Ten independent experiments were performed per single isolate.

Results

agr-Genotypes

The *agr* genotypes of all isolates were determined by the new ScaI-RFLP method. The presence of unique restriction sites for each *agr*-genotype allowed us to obtain four different restriction profiles. The amplicons of each *agr*-genotype of four selected strains, used as control, were sequenced and compared with those deposited in GenBank (Fig. 1). All 29 CVC *S. aureus* unrelated isolates fell into one of the four previously described *agr*-allele groups but in three strains PCR amplicon of unexpected size (about 4 kb) was found (Table 2). Sequence analysis of the three unexpected PCR products revealed that these isolates contained an IS*256* copy within the *agr*-locus I: in two strains, indicated as IA-variant, this element is located in the intergenic region between *agr*D and *agr*C and has the same transcription direction as the P_2 *agr*-operon at the nucleotide position 699 of the published *agr* sequence (GenBank accession no. AJ617710). In one strain (IB-variant), the insertion sequence was integrated into *agr*C and caused the interruption of this gene at nucleotide position 1114, but it had an opposite transcriptional direction with respect to the *agr*

Table 2. *agr*-Genotype distribution and strong biofilm producers in CVC and control isolates in study

	agr I		*agr* II		*agr* I+IS256		*agr* III		*agr* IV	
Strains	*n*	B-SP	*n*	B-SP	*n*	B-SP	*n*	B-SP	*n*	B-SP
$MRSA_{cvc}$ No. 17	7	–	7	7/7	3	1/3	–	–	–	–
$MSSA_{cvc}$ no.12	2	–	3	3/3	–	–	3	1/3	4	–
$MRSA_{cg}$ no.15	8	1/8	5	5/5	–	–	1	–	1	–
MSSAcg no.15	4	–	5	5/5	–	–	3	–	3	–
$MSSA_{cvc}$ no.12	2	–	3	3/3	–	–	3	1/3	4	–
$MRSA_{cg}$ no.15	8	1/8	5	5/5	–	–	1	–	1	–

Cg, control group; cvc, central venous catheter infections; B-SP, biofilm strong-producers.

genes (Fig. 2). Analysis with Sosui (http://sosui.proteome.bio.tuat.ac.jp) showed that this insertion sequence led to a truncated AgrC protein at the level of the fourth transmembrane helix.

In our strains, MRSA were grouped in *agr*-alleles I and II, both with a percentage of 41.2, and three strains (17.6%) belonging to the *agr*-allele I variant, in which the IS*256* was integrated as described below. The 12 strains of MSSA were distributed in all *agr*-types, and 16.6% were *agr*-group I, 25.0% *agr*-group II and III, and 33.3% *agr*-group IV. An overlapping distribution of the *agr*-groups was also found in the non-CVC-related strains of the control group (Table 2).

Determination of biofilm production and correlation with the *agr*-genotype

Although all *S. aureus* isolates contained the *ica*-operon and produced various amounts of biofilm, the *ica* expression – as reported in the next section – and biofilm production, were variable and tightly regulated under *in vitro* conditions. Quantitative determination of biofilm formation showed a strong biofilm production in 12 of 29 strains (eight MRSA and four MSSA). The *agr*-genotype II isolates, MRSA and MSSA, as well as one strain of agr-type III and the *agr*-IB variant were all strong biofilm producers (average value of 0.62 ± 0.177). All isolates, members of the I and IV *agr*-genotypes, showed a medium or weak capability to form biofilm on inert surfaces. In the control group, strong biofilm-producing strains were found in the same *agr*-genotypes irrespective of their isolation sites (Table 2).

Study of gene expression

All strains used in the RT-PCR experiments were positive for *ica*R, *ica*A, *agr*D, *agr*C, *agr*A, *hld*, *hla*, *sar*A, and *rsb*U at the genome level.

To examine the contribution of various genes (*agr*, *hld*, *hla*, *sar*A and *rsb*U) on *ica*-operon expression and biofilm formation, we used RT-PCR (Table 3). All strains producing a large amount of biofilm belonging to *agr*-allele II and the *agr*-IB variant expressed *ica*A at the mid-exponential and late-exponential phases ($OD_{600\,nm}$ 0.2–0.4) whereas the medium or weak biofilm producers expressed *ica*A in the late- and/or postexponential phases ($OD_{600\,nm}$ 0.4–1.0). All *agr*-II and -III isolates were defective in *agr*DCA and consequently they did not have a functional AIP – as

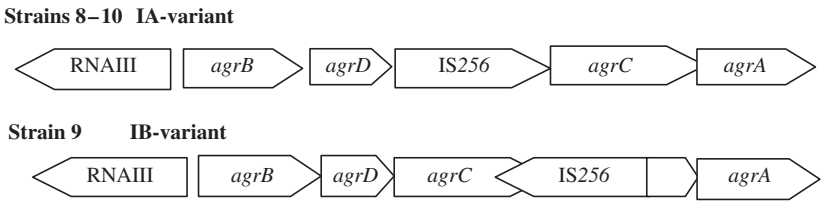

Fig. 2. IS256 integration sites.

Table 3. Study of gene expression

		Strains			P_2–RNA				P_3–RNA				
agr	Biofilm	cvc No. of isolates	cg No. of isolates	$OD_{600\,nm}$	*ica*A	*ica*R	*agr*D	*agr*C	*agr*A	*rna*III	*hla*	*sar*A	*rsb*U
II	Strong	3	3	0.2	+	–	–	–	–	–	–	+	–
				0.4	+	–	–	–	–	–	–	+	–
				1.0	–	–	–	–	–	+	–	+	–
IB	Strong	1	–	0.2	+	–	+	–	–	–	–	+	–
				0.4	+	–	+	–	–	–	–	+	–
				1.00	–	–	+	–	–	+	–	+	–
III	Medium	1	2	0.2	–	–	–	–	–	–	–	–	–
				0.4	+	+	–	–	–	+	–	+	+
				1.00	+	+	–	–	–	+	–	+	+
I	Medium	3	2	0.2	–	+	+	+	+	–	–	+	–
				0.4	+	+	+	+	+	+	+	+	+
				1.00	+	+	+	+	+	+	+	+	+
IA	Mediumweak	2	–	0.2	–	–	+	+	+	–	–	+	–
				0.4	+	+	+	+	+	+	+	+	+
				1.00	+	+	+	+	+	+	+	+	+
IV	Weak	1	2	0.2	–	–	+	+	+	+	–	+	–
				0.4	+	+	+	+	+	+	+	+	+
				1.00	–	–	+	+	+	+	+	+	–

demonstrated by the lack of transcription of the *hla* gene and the late transcription of δ-toxin (*rna*III or *hld* gene) – whereas in the *agr*-IB variant, only *agr*D was transcribed, and, due to the insertion of IS*256* in *agr*C, this mutant lacked the transcription of *agr*CA demonstrating a nonfunctional *agr* system. In the strains belonging to *agr*I-, -IV- and -IA variants, *agr*P_2–P_3 operons were always transcribed in CVC and control strains.

Expression studies were performed to document a correlation between biofilm formation, signaling via *agr* and, the role of *ica*R and two important global regulators (SarA and RsbU). SarA was involved in increasing transcription from both the P_2 and P_3 promoters and also interacted directly with the *ica* promoter inducing its transcription, whereas RsbU, a positive regulator of σ^B, was involved in the regulation of the gene expression upon changes in the environment such as starvation, heat shock, and osmotic shock (Conlon *et al*., 2004).

The six strains belonging to the *agr*-allele II did not transcribe, in any growth phase, *ica*R and the regulatory element *rsb*U, whereas they showed normal transcription of *sar*A. The *agr*-IB variant strain had a very similar transcription profile to that of the *agr*-allele II described before, in which neither *ica*R nor *rsb*U were transcribed, whereas the regulator *sar*A was transcribed. All strains belonging to *agr*III, having a nonfunctional AIP, were able to transcribe *ica*R, *sar*A and *rsb*U, though only at the late- and postexponential phases. In *agr*I-, -IV-, and -IA variant with a functional *agr*-locus, *sar*A was always expressed, whereas *rsb*U–*ica*R were not transcribed in the mid- and/or post-exponential phases.

Discussion

Intercellular signaling is involved in biofilm development by several bacteria (Davies *et al*., 1998; Ziebuhr *et al*., 1999; Huber *et al*., 2002; Li *et al*., 2002). Some information about the relationship between the *agr* system and *S. aureus* biofilm is available: the majority of the results obtained was on laboratory strains, in different biofilm growth systems, i.e. static or dynamic (Pratten *et al*., 2001; Shenkman *et al*., 2002; Yarwood *et al*., 2004), and only in a few papers was a correlation between *agr* functionality and biofilm formation demonstrated in clinical isolates (Vuong *et al*., 2000., 2003; Moore & Lindsay, 2001).

We classified our strains as belonging to one of the four *agr* groups, and, in the case of variants, by sequencing the PCR products of unexpected sizes. *Staphylococcus aureus* strains were *agr* typed by this method and the *agr* groups I and II account for about 80% of the interference groups found in MRSA strains. In fact, group I showed two additional polymorphisms: the IA variant, belonging to the same interference group I on the basis of sequence analysis, already described by Papakyriacou *et al*. (2000) and Goerke *et al*. (2003); and the IB-variant, a new variant in which the integration of IS*256* inactivated *agr*CA, influencing the functionality of the system, as determined by the late transcription of *hld* and the lack of transcription of *hla* genes.

In MSSA strains, all four *agr* alleles were uniformly distributed, including the rare *agr* group IV.

We found the same *agr*-allele distribution in both groups of microorganisms, i.e. those isolated from CVCs and those from other infection sites. In both groups of isolates we found strong biofilm producers only among those carrying the *agr*-alleles that had a nonfunctional *agr* locus and we demonstrated that this capability is independent from the isolation sites: all these strains had – potentially – the ability to form a thick biofilm layer under the right environmental conditions. The predominance of *agr* alleles I, II and the variants in type I (van Leeuwen *et al*., 2000), in our collection of *S. aureus* strains, can be explained by the well known clonal nature of MRSA. This confirms that *agr*-group distribution correlates strongly to the genetic background of the strains (Jarraud *et al*., 2002), and it may represent the first subdivision of *S. aureus* based on the fundamental biology of the organism (Projan & Novick, 1997; Novick, 2003).

All our *ica*-positive strains of *S. aureus* produced variable amounts of biofilm measured in a static system and were categorized as strong, medium and weak producers. All *agr* genotype II showed an enhanced ability to produce biofilm, and this correlation induced us to examine the mRNA production of *ica*A, *ica*R, *agr*DCA, *hld,* and *hla*, at various growth phases, including two regulators *sar*A and *rsb*U, all common genes presumably required for *S. aureus* biofilm formation in different environmental conditions (Conlon *et al*., 2002a, b; Caiazza & O'Toole, 2003; Valle *et al*., 2003). The *ica*R gene, a putative transcriptional repressor involved in environmental regulation, was never transcribed in *agr*II and -IB variant strong biofilm producers, determining an early *ica*A transcription able to explain – in part – the large amount of biofilm produced by these strains. On the other hand, in *agr*III as well as in *agr* allele I–IA–IV, medium or weak producers, the expression profile of *ica*R and *ica*A may be responsible for the basal level of biofilm produced and indicates a partial ability of *ica*R repression.

Recent papers (Valle *et al*., 2003; Resch *et al*., 2006) demonstrated that *sar*A represents an indispensable gene required in biofilm formation and in the regulation of *ica* transcription. The two contrasting hypotheses on the role of *sar*A, previously published (Pratten *et al*., 2001; Valle *et al*., 2003), have not yet been resolved. SarA was always present in our strains, demonstrating that biofilm expression seems to be unrelated to its presence and transcription; its role is not clear and needs to be further clarified.

A lack of transcription of the *agr*-locus and *hla*, or late transcription of *rna*III are also characteristics of the biofilm

producers among the agrII-, -III- and -IB variant. In all other strains, *agr*I–IA–IV, *agr* locus and the *hla* were fully expressed.

The role of δ-toxin as a surfactant able to reduce biofilm formation (as evoked by Vuong *et al.*, 2000) was confirmed in our experiments. In fact, its early transcription correlates to weak and medium biofilm producers (as in *agr*I–IA–IV). The postexponential transcription of δ-toxin correlates perfectly to the strong biofilm producers such as *agr*II.

The alternative transcription factor σ^B controls the differentiation patterns associated with starvation, other forms of stress, and the transition into the stationary phase. Conlon *et al.* (2004) demonstrated a further contribution of *rsb*U, an associated regulator to the σ^B global regulator, in determining biofilm-negative control in *S. epidermidis. rsb*U is an activator of σ^B and its mutation leads to a *sig*B deficiency. Recent data strongly suggest that RsbU and σ^B regulate important virulence factors contributing significantly to the outcome of staphylococcal infections (Jonsson *et al.*, 2004). Our results show that *rsb*U was never expressed in *agr*-genotype II and -IB variants, whereas in the other *agr*-groups it was expressed in the late- and postexponential phases. These results are, partially, in agreement with those obtained by Conlon *et al.* (2004) in *S. epidermidis*, in which transcription of *agr*P_3-driven RNAIII was substantially reduced in the *rsb*U transposon mutant M15. Our *rsb*U negative strains were deficient in *rna*II and III systems, but, contrary to what has been described in *S. epidermidis*, our strains were strong biofilm producers and not negative-variants: *rsb*U^- strains lacked α-haemolysin production and produced less δ-haemolysin. In strains in which *rsb*U was normally transcribed there was a normal level of both haemolysin transcriptions. In our study, moreover, the functionality of *rsb*U seems to be unrelated to *sar*A expression in all *agr*-types, whereas a strong correlation was demonstrated between *rsb*U and *ica*R.

In conclusion, these findings provide new insights into the variable biofilm production in clinical isolates of *S. aureus* strains. The data presented here suggest that *agr* polymorphism II and IB variants produce large amounts of biofilm, possess a defective *agr* system, and have no α-haemolysin activity, whereas δ-haemolysin is expressed only in the postexponential growth phase. The lack of *rsb*U and *ica*R transcription determines an *ica*A expression from an early growth stage. The *agr* polymorphism III strains produce a medium amount of biofilm, have an inactive *agr* system, but the *ica*AR and the regulator *rsb*U are regularly transcribed only in the late growth phase.

Biofilm production, therefore, is a dynamic process. Levels of PIA are apparently not affected by *sar*A and seem to be under the control of *agr*-types. This is coupled to an early *ica*A expression due to the lack of *rsb*U and *ica*R transcription. Further studies are necessary to identity other regulatory mechanisms in biofilm production in clinical isolates of *S. aureus.*

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