Molecular markers of quorum-sensing and virulence gene regulators in *Staphylococcus aureus* strains isolated from biofilm associated infections

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Abstract

Staphylococcus aureus is a versatile human pathogen responsible for both nosocomial and community-acquired infections. The ability of Staphylococcus (S.) aureus to cause a multitude of human infections has been ascribed to an impressive array of extracellular and cell-wall-associated virulence determinants that are coordinately expressed during different stages of infection by numerous regulatory loci, including agr, sar, sigB, sae, rot and arl (2).

The purpose of this study was to characterize at molecular level the quorum-sensing system agr and some virulence gene regulators (srrAB, sar, rot and arl) in S. aureus strains isolated from cardiovascular devices associated infections. PCR-based assays were used to evaluate agr locus nucleotide polymorphism for the identification of agr autoinducer receptor specificity groups within S. aureus strains analyzed. All isolates could be assigned to one of four major agr groups. Thus, six S. aureus strains belonged to agr group I (frequently associated with suppurative infections, like endocarditis), and three to agr group III (frequently associated with TSST-1-mediated disease).

Keywords: virulence factors, molecular markers, autoinducer, quorum-sensing, global regulators

Introduction

S. aureus is a versatile human pathogen responsible for both nosocomial and community-acquired infections. The severity of *S. aureus* – associated infections ranges from benign localized skin abscesses to life-threatening diseases such as arthritis, osteomyelitis, and endocarditis [2]. The ability of *S. aureus* to cause a multitude of human infections has been ascribed to an impressive array of extracellular and cell-wall- associated virulence determinants that are coordinately expressed during different stages of infection (i.e. colonization → avoidance of host defense → growth and cell division → bacterial spread).

The coordinated expression of many of these virulence determinants in *S. aureus* in response to environmental cues during infections (e.g. expression of adhesins early during colonization *vs*. production of toxins late in infection to facilitate tissue spread) is regulated by at least four two-component signal transduction systems, such as *agr*, *sae*, *arl* and *srrAB* and global regulators including *sar*, *sigB* and *rot* [4, 11]. These regulators are parts of an important network modulating the expression of *S. aureus* virulence genes. One target virulence gene can be under the influence of several regulators that ″cross talk″ to ensure that the specific gene is expressed only when conditions are favorable [11]. Therefore, virulence gene regulators could affect the expression of target gene directly, by binding to their promoters, or indirectly, via other regulators.

Materials and methods

i) Microbial strains

In this study, there were analyzed nine *S. aureus* strains selected from a strains pool isolated between 2005-2007 from patients hospitalized in Fundeni Hospital, Bucharest. These strains were isolated from patients with cardiovascular devices associated infections and were identified by help of conventional and API microtests and VITEK I automatic system. The phenotypic characterization for the presence of enzymatic virulence hallmarks (table 2) was established by cultivating the strains in specialized media for enzymes activity detection.

Subsequently, we used PCR amplification methods for detection of quorum-sensing system and some of the virulence gene regulators (*agr, srrAB, sarS, rot,* and *arlRS*) in these strains.

ii) Methods

PCR analysis of the QS genes

PCR assays were used for the detection of QS genetic support and virulence gene regulators in *S. aureus* strains analyzed [3]. Chromosomal DNA was extracted from the nine clinical isolates and used as templates in PCR experiments. One colony of each strain from solid medium was inoculated into 5 ml of BHI (Broth Heart Infusion) and grown overnight at 37°C with shaking. From these strain cultures DNA extraction was performed by using Wizard DNA Genomic Purification kit (Promega, USA) according to the manufacturer ′s recommendations. For the amplification of *agr* locus promoters (*agr* P_2 and *agr* P_3), the PCR primers are listed in table 1.

Table 1. Primers used for *agr* P_2 and *agr* P_3 PCR amplification.

The gene	Target	PCR primers
agr P_2	100 bp upstream of RNAII translational start site	5′-TAAAATATTAAATACAAATTACATTT-3′ 5′-ATTTTACACCACTCTCCTCA-3′
agr P_3	100 bp upstream of RNAIII translational start site	5′-TCAACTATTTTCCATCACATC-3′ 5′-ACATAAAAAAATTTACAGTTAAGA-3′

PCR for these two promoters were carried out each of them in a total volume of 50 µl with 30 ng chromosomal DNA as a template. PCR conditions for the amplification step were: 35 cycles of PCR, with 1 cycle consisting of denaturation (30 seconds at 95 °C), annealing (30 seconds at 55 °C), and extension (1.5 minutes at 72 °C) and 1 elongation cycle of 72 °C for 5 minutes. Synthesized DNA fragments were detected on 1.5% agarose gels by ethidium bromide staining. The lengths of the PCR products were estimated by comparison with the 100 bp DNA ladder molecular size markers (Promega).

The *agr* specificity groups were identified by PCR amplification of the hypervariable domain of the *agr* locus using oligonucleotide primers specific for each of the four major specificity groups (fig. 1).

A forward primer, pan-*agr* (5′-ATGCACATGGTGCACATGC-3′), corresponding to the conserved sequences from the *agrB* gene, was used in all reactions (Fig. 1) (primer sequences were obtained from GenBank accession numbers X52543, AF001782, AF001783, and AF288215) [12]. Four reverse primers, each specific for amplification of a single *agr* group based on *agrD* or *agrC* gene nucleotide polymorphism, were as follows: *agr* I, 5′-GTC ACAAGTACTATAAGCTGCGAT-3′ (in the *agrD* gene); *agr* II, 5′-GTATTACTAATTGAAAAGTGCCATAGC-3′ (in the *agrC* gene); *agr* III, 5′-CTGTTGAAAAAGTCAACTAAAAGCTC-3′ (in the *agrD* gene); and *agr* IV, 5′-CGATAATGCCGTAATACCCG-3′ (in the *agrC* gene). The *agr* specificity groups were identified by the expected product sizes (*agr* I – 440 bp, *agr* II – 572 bp, *agr* III – 406 bp si *agr* IV – 588 bp).

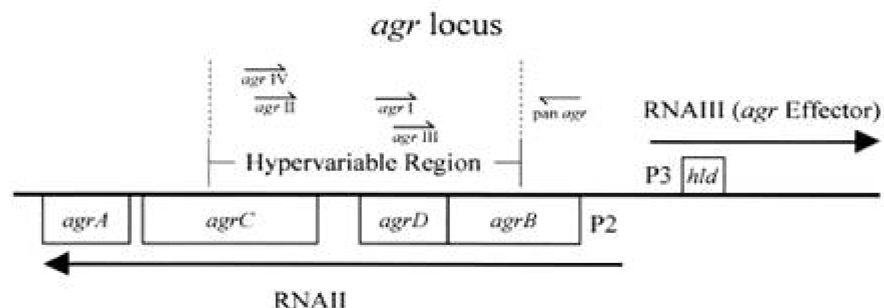

Figure 1. Schematic map of the *S. aureus agr* locus showing the locations of the different primers used for amplification of the hypervariable region (after Shopsin, 2003).

The *agr* locus in *S. aureus* has been shown to be polymorphic, because consists from highly conserved and hypervariable regions among *S. aureus* strains, and can be divided into four distinct genetic groups. The sequence of this hypervariable segment is the target of PCR amplification for defining *agr* groups. PCR for *agr* specificity groups identification was performed by adding 4 µl of chromosomal template DNA and 17.25 µl of water to 50 µl of a PCR mixture that includes 5 U of GoTaq Flexi DNA polymerase, 25 mM MgCl2, 10 mM

(total) deoxynucleoside triphosphates, in 0.5-ml PCR tubes in a Thermo Cycler (Biorad-MJ). A negative control (pure water) was included. For gene amplicons PCR conditions for the amplification step were: 1 cycle consisting of denaturation (1 min. at 94°C), annealing (1 min. at 55°C), extension (1 min. at 72°C), and 1 elongation cycle of 72 °C for 5 minutes. PCR products were separated by electrophoresis on 1.5% agarose gels, which were stained with ethidium bromide. The lengths of the PCR products were estimated by comparison with the 100 bp DNA ladder molecular size markers (Promega) (Fig. 2, 3, 4).

PCR analysis for detection of the virulence gene regulators

The PCR conditions for the amplification of *sarS* gene and *rot* locus were: 1 cycle consisting of denaturation (1 min. at 94°C), annealing (1 min. at 55°C), extension (1 min. at 72°C), and 1 elongation cycle of 72 °C for 5 minutes.

The PCR conditions for the amplification of promoter of *srrAB* locus were: 1 cycle consisting of denaturation (1 min. at 94°C), annealing (1 min. at 50°C), extension (1 min. at 72°C), and 1 elongation cycle of 72 °C for 5 minutes. The target of amplification is a sequence of 100 bp upstream of translational start site.

For PCR amplification of *arlRS* locus the conditions were the same with those for promoter of *srrAB* locus with a single difference (the final elongation cycle was 10 minutes at 72 °C). The specific primers for *sarS*, *rot*, *srrAB* and *arlRS* genes amplification are listed in table 2. PCR products for all these genes were separated by electrophoresis on 1.5% agarose gels, which were stained with ethidium bromide. The lengths of the PCR products were estimated by comparison with the 100 bp DNA ladder molecular size markers (Promega) (Fig. 5, 6, 7).

Table 2. Primers used for the amplification of *sarS*, *rot*, *srrAB* and *arlRS* genes.

Gene	Forward primer	Reverse primer
sarS	5′-AGTTTTATGTTATAAACAATCGGA-3′	5′-AGTTTTATGTTATAAACAATCGGA-3′
rot	5′-GTTTTGGGATTGTTGGGATG-3′	5′- GCATTGCTGTTGCTCTACTTGC-3′
srrAB	5′-ACAGGTCATACCTCCCAC-3′	5′- AGAATTTTTTCACAAAATTTTAG-3′
arlRS	5’-AATTTTACGTCGTCAGCCACAA-3’	5’- GATTTTTAGATGTCAGATCCGT-3’

Results

The aim of this study was to determine the *agr* specificity groups and the genes encoding the virulence global regulators (*sarS*, *rot*, *srrAB* and *arlRS*) in *S. aureus* strains analyzed.

The expression of soluble, enzymatic factors (haemolytic activities, lecithinase, lipase, caseinase, amylase, DN-ase) in *S. aureus* strains was assayed by spot plate method using specific media, as previously described [1, 7].

No.	***S. aureus* strains** isolated from blood cultures	**Lecithinase**	**Lipase**	**Amylase**	**DN-ase**	**Caseinase**	**β - hemolysin**	**Camp factor**
1	10936	++	-	-	++	+++	-	+
2	11372	++	+++	-	+++	+++	++	+
3	11327	++	++	-	+++	+++	-	+
4	MRSA 11325	++	+++	-	+++	+++	++	+
5	MRSA 11573	-	-	-	+++	+++	-	+
6	MRSA 11047	+	-	-	+++	+++	-	+
7	5/06	-	-	-	+++	++	-	+
8	9/06	+±	++	-	+±	+++	+	+
9	11323	++	+++	-	+++	++	++	+

The results obtained shows that the strains analyzed possesses some of these soluble, enzymatic factors (lecithinase, lipase, amylase, DN-ase, caseinase and haemolysins) that plays important roles during cardiovascular devices associated infections.

Figure 2. Gel electrophoresis of amplified products of promoter *agr* P2. Lines 1 – DNA ladder 100bp, 2 - negative *S. aureus* strain, 3 - *S. aureus* 10936, 4 - *S. aureus* 11372, 5 - *S. aureus* 11327, 6 - MRSA 11325, 7 - MRSA 11573, 8 - MRSA 11047, 9 - *S. aureus* 5/2006, 10 - *S. aureus* 9/2006, 11 - DNA ladder 100bp, 12 - *S. aureus* 11323.

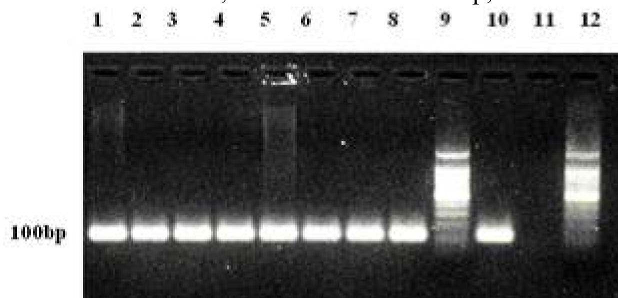

Figure 3. Gel electrophoresis of amplified products of promoter *agr* P3. Lines 1 - *S. aureus* 10936, 2 - *S. aureus* 11372, 3 - *S. aureus* 11327, 4 - MRSA 11325, 5 - MRSA 11573, 6 - MRSA 11047, 7- *S. aureus* 5/2006, 8 - *S. aureus* 9/2006, 9 - DNA ladder 100bp, 10 - *S. aureus* 11323, 11 - negative *S. aureus* strain, 12 – DNA ladder 100bp.

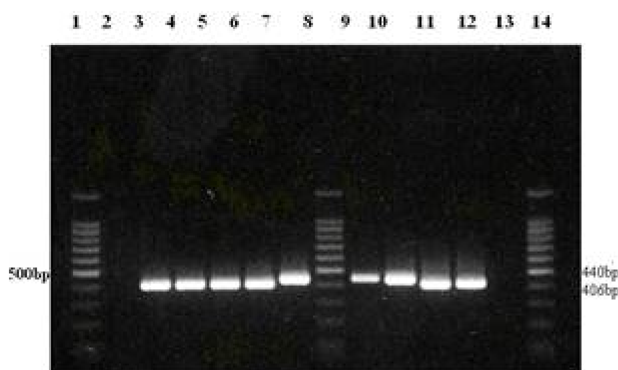

Figure 4. Gel electrophoresis of amplified products of *agr* gene. Lines 1 - DNA ladder 100bp, 2 - negative *S. aureus* strain, 3 - *S. aureus* 10936, 4 - *S. aureus* 11372, 5 - *S. aureus* 11327, 6 - MRSA 11325, 7 - MRSA 11573, 8 - DNA ladder 100bp, 9 - MRSA 11047, 10 - *S. aureus* 5/2006, 11 - *S. aureus* 9/2006, 12 - *S. aureus* 11323, 13 – pure water, 14 – DNA ladder 100bp.

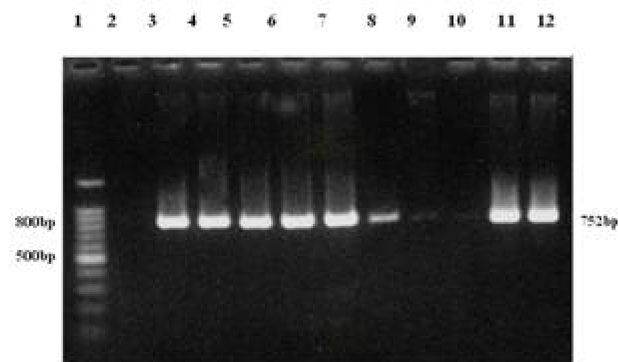

Figure 5. Gel electrophoresis of amplified products of *sarS* gene. Lines 1 – DNA ladder 100bp; 2 – negative *S. aureus* strain, 3 - *S. aureus* 10936, 4 - *S. aureus* 11372, 5 - *S. aureus* 11327, 6 - MRSA 11325, 7 - MRSA 11573, 8 - MRSA 11047, 9 - *S. aureus* 5/2006, 10 - pure water, 11 –*S. aureus* 9/2006, 12 - *S. aureus* 11323.

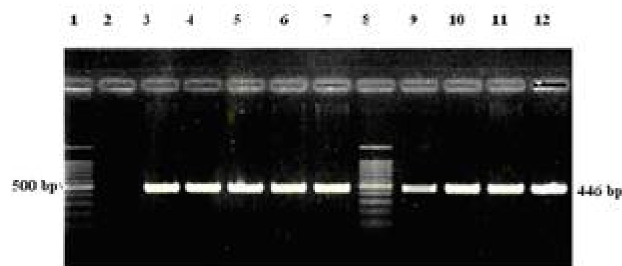

Figure 6. Gel electrophoresis of amplified products of *rot* gene. Lines 1 – DNA ladder 100bp, 2 – negative *S. aureus* strain, 3 - *S. aureus* 10936, 4 - *S. aureus* 11372, 5 - *S. aureus* 11327, 6 - MRSA 11325, 7 - MRSA 11573, 8 - DNA ladder 100bp, 9 - MRSA 11047, 10 - *S. aureus* 5/2006, 11 - *S. aureus* 9/2006,12 - *S. aureus* 11323.

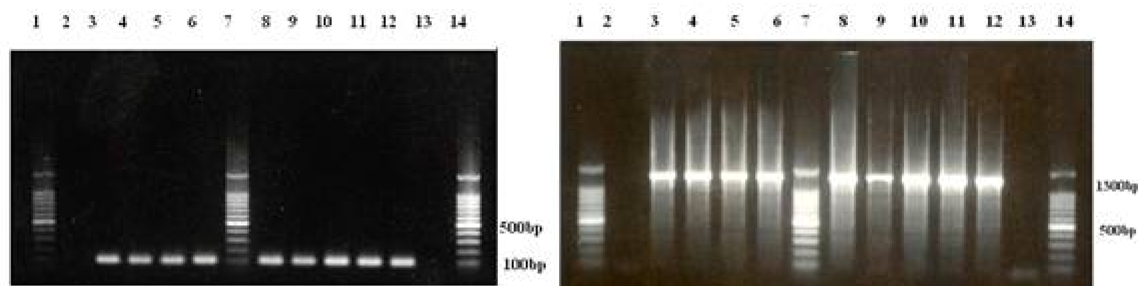

Figure 7. Gel electrophoresis of amplified products of the promoter *srrAB* and *arl* locus. Lines 1 – DNA ladder 100bp, 2 – negative *S. aureus* strain, 3 - *S. aureus* 10936, 4 - *S. aureus* 11372, 5 - *S. aureus* 11327, 6 - MRSA 11325, 7 – DNA ladder 100bp, 8 - MRSA 11573, 9 - MRSA 11047, 10 - *S. aureus* 5/2006, 11 - *S. aureus* 9/2006, 12 - *S. aureus* 11323, 13 - pure water, 14 - DNA ladder 100bp.

Discussion

The coordinated expression of *S. aureus* virulence factors is regulated by a complex network that includes QS system *agr* and well characterized virulence gene regulators.

The **accessory gene regulator (*agr)*** is a QS system activated by autoinducing peptide pheromone (AIP), being involved in globally control of the coordinated production of virulence factors. The *agr* locus consists of two divergent transcriptional units, RNAII and RNAIII, which are under the control of the P_2 and P_3 promoters, respectively [10]. RNA II is a polycistronic mRNA that encodes the *agrB* and *agrD* genes required for the synthesis of the AIP and also the two-component signal transduction proteins, AgrA and AgrC, which are responsible for sensing and responding to the AIP. RNA III, the effector molecule of the *agr* response, is acting primarily at the level of gene transcription, encodes the toxin δ–hemolysin (via *hld*), but increases the transcription and in some cases, translation of several secreted virulence factors, including TSS toxin-1 and other hemolysins.

The expression of many *S. aureus* virulence factors is under the control of the *agr*, which, on entering post-exponential phase, downregulates the production of cell-surface-associated proteins (protein A and coagulase) and upregulates the expression of secreted proteins (e.g., lipase, hemolysins, and proteases), that are postulated to play a role in tissue invasion and toxins (toxic shock syndrome toxin I) [10].

A polymorphism in the amino acid sequence of the autoinducing peptide and of its corresponding receptor (AgrC) has been described. *S. aureus* strains can be divided into four major groups on this basis: within a given group, each strain produce a peptide that can activate the *agr* response in the other member strains, whereas the autoinducing peptides produced by the different groups are usually mutually inhibitory. Recent studies have shown the existence of a strong association between the *agr* types and certain of *S. aureus* diseases [6]. Most ET - producing strains belong to *agr* group IV, TSST-1-mediated infections belong to *agr* group III, bullous impetigo strains are associated with *agr* groups II and IV, and suppurative infections (endocarditis) belong to *agr* groups I and II.

The *sar* (staphylococcal accessory regulator) is one of the most important virulence gene regulator because is required for the full *agr* transcription, being at least partly involved in the up-regulation of transcription from the $agrP_2$ and $agrP_3$ promoters. This global regulatory locus contained within a 1.2-kb fragment, encodes a 372-bp open reading frame with three upstream promoters driving three overlapping transcripts, each coding for the 14.5-kDa SarA protein [5]. In contrast to *agr*, the *sar* locus up-regulates the expression of many cell wall proteins (e.g., fibronectin binding protein A, fibrinogen-binding proteins) and secreted exoproteins (e.g., α- [*hla*], β– [*hlb*] and δ- hemolysins), while

repressing the transcription of the protein A gene (*spa*), collagen-binding protein (*cna*), and serine proteinase (*sspA*) [8]. SarA is the major regulatory molecule of this locus, and mediates its effect both directly by binding to target gene promoters (e.g. *agr, hla* and *spa*) and indirectly *via* the downstream effect on other regulons. The expression of SarA peaks during late exponential phase and coincides with activation of *agr* during the postexponential phase, presumably *via* SarA-*agr* promoter interaction. A number of *sarA* homologues, including *sarT* and *sarS,* have been identified in the *S. aureus* genome. All the SarA homologues act as global regulators of virulence genes. Previous studies have shown that *sarA* influences expression of both *sarT* and *sarS* in the global regulatory network. *In vitro* studies have demonstrated that *agr* negatively regulates the expression of *spa*, encoding protein A, whereas SarS binds to the *spa* promoter and activates its expression. The *agr* downregulates *sarS* expression; thus has been proposed that *agr* downregulates *spa* expression by downregulating the expression of its activator, *sarS* [2].

Rot (repressor of toxins), a SarA homologue, is a global regulator that negatively regulates the transcription of virulence genes that encodes secreted proteins (e.g., lipase, hemolysins, and proteases), and also positively regulates the expression of genes encoding some cell surface adhesins, such as ClfB (clumping factor B), and SdrC, *spa* gene, that encodes protein A, and *dltB*, member of *dlt* operon that confer resistance to defensins, human antimicrobial agents. Rot positively regulates the expression of *sarS* and may affect *spa* gene transcription *via* SarS.

The global regulators Rot and *agr* have opposing effects on the expression of certain virulence determinants. Thus, Rot promotes early stages of *S. aureus* infections when adhesins allow the bacteria to attach to host cells, and protein A and Dlt A, -B, -C and –D facilitate establishment of the infection through inhibition of the host immune response. During late stages of infection, which correspond to the post-exponential growth phase, there is a peak of the levels of RNA III, the *agr* effector molecule. Since *rot* is transcribed throughout the growth curve, Rot is likely to be inhibited by RNAIII post-transcriptionally. The inhibitory effect of RNA III on Rot result in the repression of the cell surface proteins and the activation of the secreted proteins that promotes the spread of the bacteria and allow them to initiate infection at other sites [11].

SrrAB (staphylococcal respiratory response AB), a two-component system, plays a role in virulence factors regulation in response to oxygen availability. The srrAB locus consists by two predicted open reading frames that overlap by 20 bp. *srrA* encode a response regulator, while *srrB* encode a histidin kinase. This system down-regulates production of *agr* RNAIII, protein A, and toxic shock syndrome toxin 1 (TSST-1), particularly under low-oxygen conditions. The ability of SrrAB system to regulate virulence factors and regulatory elements, such as *agr* RNAIII, protein A, TSST-1, and SrrAB is likely due its ability to bind the *agr, spa, tst* and *srr* promoters [9]. Therefore, SrrAB may modify virulence factor expression through *agr* dependent and *agr*-independent mechanisms.

ArlRS is a global two-component virulence regulator which directly and/or indirectly interacts with other regulators in regulatory networks and modulates the expression of genes involved in autolysis, cell division, growth and pathogenesis [13].

The results of this study show that six *S. aureus* strains belonged to *agr* group I (frequently associated with suppurative infections, like endocarditis), and three to *agr* group III (frequently associated with TSST-1-mediated disease). In addition, these strains analyzed possess all the virulence gene regulators analyzed. This study is demonstrating the necessity of functional QS systems for the initiation of an infectious process by opportunistic bacteria, highlighting their utility in the development of new ecologic anti-infectious strategies, based on the use of QS antagonists.

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