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Impact of *sar* and *agr* on methicillin resistance in *Staphylococcus aureus*

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Abstract

The global regulators *agr* and *sar* control expression of cell wall and extracellular proteins. Inactivation of either *sar* and/or *agr* in a typical heterogeneously methicillin-resistant *Staphylococcus aureus* resulted in a small but reproducible decrease in the number of cells in the subpopulation expressing high methicillin resistance. The amount of low affinity penicillin-binding protein PBP2′, the prerequisite for methicillin resistance, was apparently not affected, however, a reduction in PBP1 and PBP3 production was observed, suggesting that these resident PBPs of the cells might be involved somehow together with PBP2′ in high level methicillin resistance.

Keywords: Global regulator; Penicillin-binding protein; PBP1; PBP3; *mecA*; *Staphylococcus aureus*

1. Introduction

Penicillin-binding proteins catalyze in *Staphylococcus aureus* the transpeptidation and carboxypeptidation of the bacterial cell wall peptidoglycan. In methicillin-resistant *S. aureus* an additional, low affinity penicillin-binding protein PBP2′ allows survival of the cells in the presence of methicillin concentrations that saturate and inhibit the resident staphylococcal PBPs. Any alterations in peptidoglycan precursor formation leads to suboptimal function of PBP2′, resulting in a reduction of peptidoglycan cross-linking and an increase in methicillin susceptibility (reviewed in [1]). Moreover, various external factors such as pH, osmolarity, O_2 tension, and temperature are known to modulate resistance levels. This multiple dependence of methicillin resistance opens the possibility that it might be influenced in some way, maybe indirectly, by global regulators that control the activities of unlinked genes in response to certain environmental signals. At least three global regulatory systems including *agr*, *xpr* and *sar* [2–4] are involved in regulation of virulence determinants and surface protein synthesis in *S. aureus*. The polycistronic locus *agr* has a growth phase-dependent control on toxin and exoprotein production [2]. Experimental evidence suggests that *xpr* and *agr* are possibly interactive regulatory genes [4]. The third locus, *sarA* [3], was shown to be necessary for the optimal expression of *agr* [5] by acting on the RNAII promoter which controls *agr*-related transcription [6]. Diminished virulence of *sar* and/or *agr* mutants in experimental rabbit endocarditis sug-

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gests that both the *sar* and *agr* loci are involved in initial valvular adherence, intravegetation persistence and multiplication of *S. aureus* in endocarditis [7]. In this study we investigated the influence of *sar* and *agr* on the expression of methicillin resistance in *S. aureus*.

2. Materials and methods

2.1. Bacteria, phages and culture conditions

The strains used in this study are listed in Table 1. Growth was in LB broth (10 g/l of tryptone, 5 g/l of yeast extract and 5 g/l of NaCl) at 37°C unless mentioned otherwise. Transductions were done with phage 85 [8] by selecting transductants on erythromycin 20 μg/ml or tetracycline 5 μg/ml. Population analysis profiles were established by plating aliquots of an overnight culture on increasing concentrations of methicillin and determining the colony-forming units (cfu) after 48 h incubation at 35°C. Minimal inhibitory concentrations (MICs) of antibiotics were determined by the E-test (AB Biodisk, Solna, Sweden) [9]. Antibiotic gradient plates were inoculated

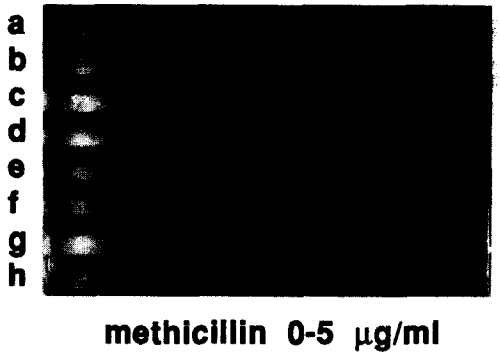

Fig. 1. Effect of *sar* and *agr* on methicillin resistance. The strains were applied along the plates containing a gradient from 0 to 5 μg/ml of methicillin. Lanes: a, Mc^r strain BB270; b, its *sar* mutant BB1030; c, its *agr* mutant BB1165; d, its *sar-agr* double mutant BB1166. Lanes: e, Mc^r penicillinase producing strain BB565; f, its *sar* mutant BB1032; g, its *agr* mutant BB1167; h, its *sar-agr* double mutant BB1168.

Table 1
Staphylococcus aureus strains

Strain	Relevant genotype (all strains are derived from NCTC8325)	Relevant phenotype	Origin/Reference
A	*sar*::Tn*917*LTV1	Em^r	[5]
RN6911	Δ*agr*::*tet*	Tc^r	[2]
BB255		Mc^s	[16]
BB1038	*sar*::Tn*917*LTV1	Mc^s, Em^r	this study, by transduction of *sar*::Tn*917*LTV1 from strain A into BB255
BB1163	Δ*agr*::*tet*	Mc^s, Tc^r	this study, by transduction of Δ*agr*::*tet* from strain RN6911 into BB255
BB1164	Δ*agr*::*tet*, *sar*::Tn*917*LTV1	Mc^s, Tc^r, Em^r	this study, by transduction Δ*agr*::*tet* from strain RN6911 into BB1038
BB270	*mec*	Mc^r	[16]
BB1030	*mec*, *sar*::Tn*917*LTV1	Mc^r, Em^r	this study, by transduction of *sar*::Tn*917*LTV1 from strain A into BB270
BB1165	*mec*, Δ*agr*::*tet*	Mc^r, Tc^r	this study, by transduction of Δ*agr*::*tet* from strain RN6911 into BB270
BB1166	*mec*, Δ*agr*::*tet*, *sar*::Tn*917*LTV1	Mc^r, Tc^r, Em^r	this study, by transduction Δ*agr*::*tet* from strain RN6911 into BB1030
BB565	*mec*, pI524	Mc^r, Amp^r	[17]
BB1032	*mec*, pI524, *sar*::Tn*917*LTV1	Mc^r, Amp^r, Em^r	this study, by transduction of *sar*::Tn*917*LTV1 from strain A into BB565
BB1167	*mec*, pI524, Δ*agr*::*tet*	Mc^r, Amp^r, Tc^r	this study, by transduction Δ*agr*::*tet* from strain RN6911 into strain BB565
BB1168	*mec*, pI524, Δ*agr*::*tet*, *sar*::Tn*917*LTV1	Mc^r, Amp^r, Em^r, Tc^r	this study, by transduction Δ*agr*::*tet* from strain RN6911 into BB1032

Abbreviations: Amp, ampicillin; Em, erythromycin; Mc, methicillin; Tc, tetracycline; ^{r}, resistant; ^{s}, susceptible.

with a suspension of 5×10^7 cells/ml applied with a cotton swab along the gradient, and incubated for 18 h at 35°C.

2.2. Labelling and detection of the PBPs

Membrane proteins were prepared from overnight cultures grown at 30°C in LB medium by differential centrifugation as described earlier [10]. 30 μg of protein in 10 μl phosphate buffer (50 mM, pH 7) was labelled at 30°C for 10 min with a final concentration of 10 μg/ml of [^{3}H]benzylpenicillin (ca. 670 GBq/mmol, Amersham) and separated on 30% acrylamide-0.8% bisacrylamide gels. The PBPs were visualized by fluorography. To determine the PBP2′ concentration by Western blots, membrane proteins were transferred to a nitrocellulose filter and probed with monoclonal mouse antibodies directed against PBP2′. Unspecific binding of the antibody to protein A was blocked by preincubation with human IgG (Strandén, personal communication). The bound PBP2′ antibody was detected with a secondary goat anti-mouse antibody horseradish peroxidase conjugate from BioRad and visualized with 4 chloro-naphthol in methanol.

3. Results

A set of isogenic *sar* and *agr* mutants were constructed by phage 85-mediated transduction using susceptible strain BB255, and its isogenic methicillin-resistant (Mc^r) derivatives BB270 and BB565,

Table 2
Minimal inhibitory concentrations (μg/ml) determined by E-test

Strain	Oxacillin	Cefoxitin	Imipenem
BB270	>256	96	12
BB1030	192	16	4
BB1165	256	24	0.75
BB1166	128	6	0.25
BB565	48	32	0.094
BB1032	8	8	0.094
BB1167	6	16	0.125
BB1168	6	8	0.064
BB255	0.25	3	0.032
BB1038	0.25	2	0.032
BB1163	0.19	3	0.023
BB1164	0.25	2	0.023

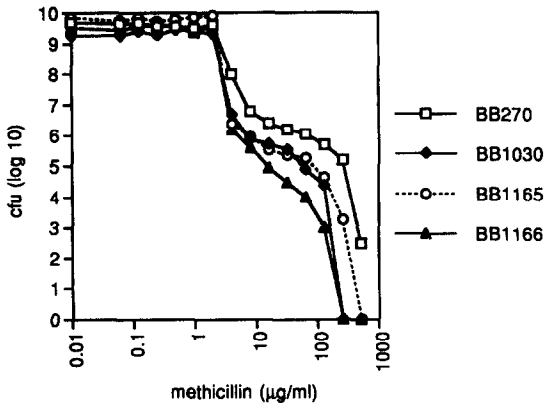

Fig. 2. Effect of *sar* and *agr* on the resistance profile of Mc^r strain BB270. Population analyses were made from Mc^r strain BB270, its *sar* mutant strain BB1030, its *agr* mutant BB1165 and its *sar-agr* double mutant BB1166 by plating aliquots of an overnight culture on increasing concentrations of methicillin. The colony-forming units (cfu) were determined after 48 h incubation at 35°C.

the latter a Mc^r strain that carries a penicillinase plasmid, as recipients. The resulting transductants were the *sar* mutants BB1038, BB1030 and BB1032, and the *agr* mutants BB1163, BB1165 and BB1167. Double mutants were obtained in a second step by transduction of the *agr* null mutation into the respective *sar* mutants, yielding the corresponding double mutants BB1164, BB1166 and BB1168.

Inactivation of *sar* or *agr* increased the minimal doubling time of Mc^r parent strain BB270 in LB medium from 27 min to 33 min in BB1030 and BB1165. The effect was additive in the double mutant BB1166 which had a doubling time of 36 min. All strains reached the same optical density after 24 h growth, corresponding to approximately 5×10^9 cfu/ml.

There was a reproducible general decrease in β-lactam resistance in the *sar* and *agr* mutants, which was more pronounced in the double mutants (Table 2). The *sar* and *agr* effects were qualitatively similar in the mutants constructed in either Mc^r BB270 or in the Mc^r penicillinase-producing strain BB565, whereas no effect was measurable in the Mc^s parent BB255. This drop in methicillin resistance between the strains could be made clearly apparent on plates containing a gradient of methicillin as shown in Fig. 1. Similar results were obtained with cefoxitin (data not shown). Noteworthy is that penicillinase produc-

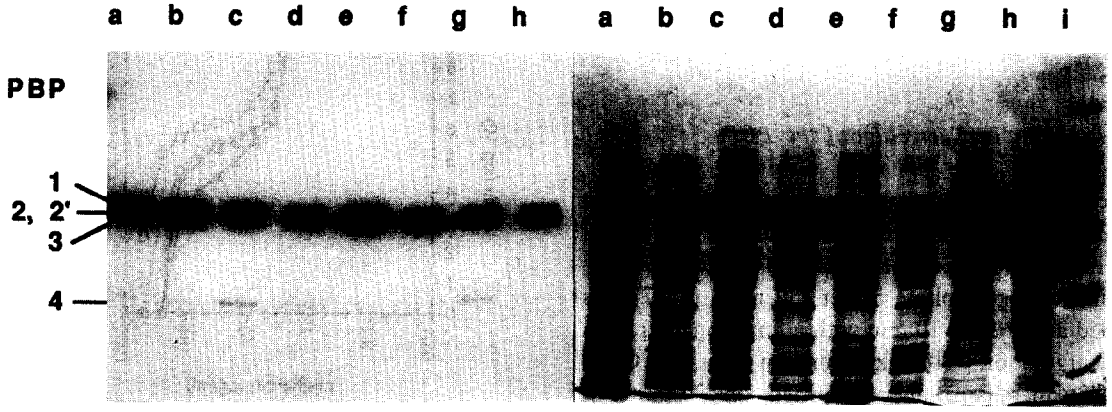

Fig. 3. Effect of *sar* and *agr* on PBPs and membrane proteins separated on a polyacrylamide gel. (A) Autoradiograph of [^{3}H]penicillin labelled PBPs. (B) Coomassie blue-stained membrane proteins. Lanes: a, susceptible parent strain BB255; b, its *sar* mutant BB1038; c, its *agr* mutant BB1163; d, its *sar-agr* double mutant BB1164. Lanes: e, Mc^r strain BB270; f, its *sar* mutant BB1030; g, its *agr* mutant BB1165; h, its *sar-agr* double mutant BB1166. Lane i, molecular weight marker.

tion in strain BB565 resulted in a generally lower methicillin resistance compared to BB270, the penicillinase-free isogenic Mc^r parent, by both methods.

Typical for Mc^r strains is the heterogeneous expression of the methicillin resistance. The basal resistance of the heterogeneously Mc^r strain BB270 was rather low but a subpopulation of approximately one cell in 10^4 was resistant to much higher concentrations of methicillin (Fig. 2). Inactivation of either *sar* or *agr* had no effect on the basal resistance but reduced the number of cells able to form the highly resistant subpopulation by a factor of 10 approximately. Whereas BB270 still produced over 10^2 cfu/ml in the presence of 512 μg/ml of methicillin, the *agr* mutant BB1165 did not grow at that concentration any more and formed colonies only up to 256 μg/ml. The highly resistant subpopulation of *sar* mutant BB1030 was even more susceptible and grew up to only 128 μg/ml of methicillin. The double mutant, finally, BB1166, was as susceptible as the *sar* mutant, with approximately a 10-fold smaller highly resistant subpopulation than that of the single mutants.

A rather remarkable overall difference in membrane protein pattern was notices, elicited mainly by the inactivation of *sar* (Fig. 3B, lanes b, d, f and h). When analyzing the effects of *sar* and/or *agr* on the PBP profiles of Mc^s strain BB255 or Mc^r strain BB270, there seemed to be slightly less PBP1 mainly in the *sar* mutants BB1033 and BB1030, whereas in the *agr* mutants BB1163 and BB1165 there was less PBP3 compared to the respective parent strains BB255 and BB270 (Fig. 3A). In the double mutants BB1164 and BB1166 the effects were cumulated. The amount of PBP2 in strain BB255 as well as the combined amounts of PBP2 and PBP2′ in BB270, which comigrated in these gels, were apparently not affected in the mutants. A Western blot done with antibodies directed against PBP2′ showed no relevant differences in PBP2′ content in the mutants derived from the Mc^r strain BB270 (Fig. 4).

4. Discussion

The proportion of the highly methicillin-resistant minority as well as their maximal resistance was reduced by *sar* and/or *agr* inactivation in BB270, whereas the basal methicillin resistance remained essentially unaltered. The high and constitutive production of PBP2′ was not affected, but the membrane protein pattern was altered and unexpectedly the PBP1 and PBP3 content seemed to be reduced, as could also be observed in the BB255-derived mutants. At which step or in what way the global regulators downregulate the apparent PBP content remains to be analyzed. Whether the reduction in the amount of resident PBPs was the cause of the small

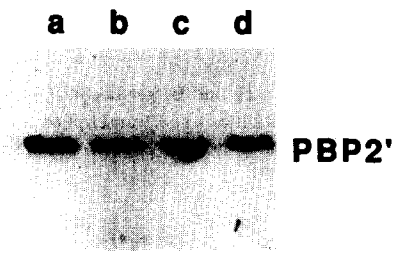

Fig. 4. Effect of *sar* and *agr* on low affinity PBP2′. Western blot of PBP2′. Lanes: a, Mc^r strain BB270; b, its *sar* mutant BB1030; c; its *agr* mutant BB1165; d, its *sar-agr* double mutant BB1166.

but reproducible reduction in resistance is an interesting and intriguing hypothesis, which would assign a role to the resident PBPs besides PBP2′ in methicillin resistance. High methicillin resistance was shown to depend on chromosomal genes [11]. The contribution of the normal staphylococcal PBPs to methicillin resistance in a Mc^r background has not yet been investigated systematically. PBP4 overproduction [12], or alterations in the amount and/or kinetics of PBP2 and/or PBP4 [13,14] may contribute to increased resistance, and low level methicillin-resistant clinical isolates with altered methicillin binding affinities of PBP1 and PBP2 and PBP4 overproduction have been reported [15]. There might be an additive effect between the staphylococcal PBPs and PBP2′ in methicillin resistance.

It would be interesting to know if any qualitative differences existed in the expression of the global regulators in different genetic backgrounds, especially in highly homogeneously Mc^r clinical isolates and their heterogeneously resistant counterparts, and if other target genes besides cell wall and extracellular proteins are controlled by the *agr-sar* regulators.

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