

# Biofilm density and detection of biofilm-producing genes in methicillin-resistant *Staphylococcus aureus* strains

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**Abstract** Many serious diseases caused by *Staphylococcus aureus* appear to be associated with biofilms. Therefore, we investigated the biofilm-forming ability of the methicillin-resistant *S. aureus* (MRSA) isolates collected from hospitalized patients. As many as 96 % strains had the ability to form biofilm in vitro. The majority of *S. aureus* strains formed biofilm in *ica*-dependent mechanism. However, 23 % of MRSA isolates formed biofilm in *ica*-independent mechanism. Half of these strains carried *fnbB* genes encoding surface proteins fibronectin-binding protein B involved in intercellular accumulation and biofilm development in *S. aureus* strains. The biofilm structures were examined via confocal laser scanning microscopy (CLSM) and three-dimensional structures were reconstructed. The images obtained in CLSM revealed that the biofilm created by *ica*-positive strains was different from biofilm formed by *ica*-negative strains. The MRSA population showed a large genetic diversity and we did not find a single clone that occurred preferentially in hospital environment. Our results demonstrated the variation in genes encoding adhesins for the host matrix proteins (elastin, laminin, collagen, fibronectin, and fibrinogen) and in the gene involved in biofilm formation (*icaA*) within the majority of *S. aureus* clones.

## Abbreviations

Bap	Biofilm-associated protein
CLSM	Confocal laser scanning microscopy
CRA	Congo red agar
FNBP	Fibronectin-binding protein B

MLVA	Multiple-locus variable-number tandem repeat analysis
MPU S	Bacteriology Collection of Department of Microbiology A. Mickiewicz University, Poznań
MRSA	Methicillin-resistant <i>S. aureus</i>
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
PCR	Polymerase chain reaction
PI	Propidium iodide
PIA	Polysaccharide intercellular adhesion

## Introduction

*Staphylococcus aureus* is a Gram-positive bacterium responsible for many infections ranging from folliculitis to septicemia, pneumonia, osteomyelitis, or endocarditis (Götz et al. 2006). Many infections are mediated by the ability of *S. aureus* to adhere to catheters and other medical devices and form multicellular communities embedded in polymeric matrix known as the biofilm. Treatment of *Staphylococcus*-biofilm-associated infections is extremely difficult because inside the biofilm, *S. aureus* becomes more resistant to antibiotic treatment and the action of the host immune system. Biofilm formation is a complex process that can be subdivided into relatively distinct phases: primary attachment of cell, cell-to-cell adhesion and proliferation, biofilm maturation, and finally detachment of planktonic cell from the biofilm (Götz 2002; Mack et al. 2006). Several cell surface proteins of *S. aureus* referred to as MSCRAMMs have been implicated in primary attachment to both native tissues and abiotic surface. Having successfully attached itself, the bacteria start to accumulate in multiple bacterial layers and construct a multilayered archi-

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ture. The accumulative phase is linked to the production of polysaccharide intercellular adhesin (PIA) which is composed of linear  $\beta$ -1,6-linked glucosaminylglycans. PIA is synthesized by the products of *ica* locus, particularly by the enzyme *N*-acetylglucosaminyltransferase encoded by the *icaA* gene (Mack et al. 2006). The *ica*-dependent biofilm formation is the main mechanism described in *S. aureus* strains (Mack et al. 2006). Recently, O'Neill et al. (2007) have indicated that deletion of the *ica* locus has no effect on biofilm formation in some methicillin-resistant *S. aureus* (MRSA) strains. Intercellular adhesion and biofilm development in *S. aureus* strains are mediated by a 140-kDa cell wall surface protein biofilm-associated protein (Bap) (Cucarella et al. 2001). Additional components such as proteins other than Bap, DNA, or teichoic acid have also been suggested to be important in biofilm formation. After biofilm maturation, the detachment of cell from the biofilm may initiate a new cycle of biofilm formation elsewhere. Therefore, the ability of *S. aureus* strains to form biofilm is considered an important virulence factor in the establishment of chronic infections.

The purpose of this study was to examine the ability of methicillin-resistant *S. aureus* strains to produce slime and form biofilm in vitro. The biofilm structures were examined via confocal laser scanning microscopy. Moreover, we estimated the prevalence of genes encoding adhesins for the host matrix proteins (elastin, laminin, collagen, fibronectin, fibrinogen, and bone sialoprotein) and genes involved in biofilm formation (*icaA* and *bap*). To assess the epidemiological relation of MRSA strains we used multiple-locus variable-number tandem repeat analysis (MLVA).

## Material and methods

**Bacterial strains** Eighty MRSA were collected from treated patients in a large hospital in Poznań for 4 years (Table 1.). The bacteria were identified by conventional methodology and using Vitek 2 system (bioMérieux, France). All isolates

were resistant to oxacillin, which was confirmed by the presence of *mecA* gene (Geha et al. 1994).

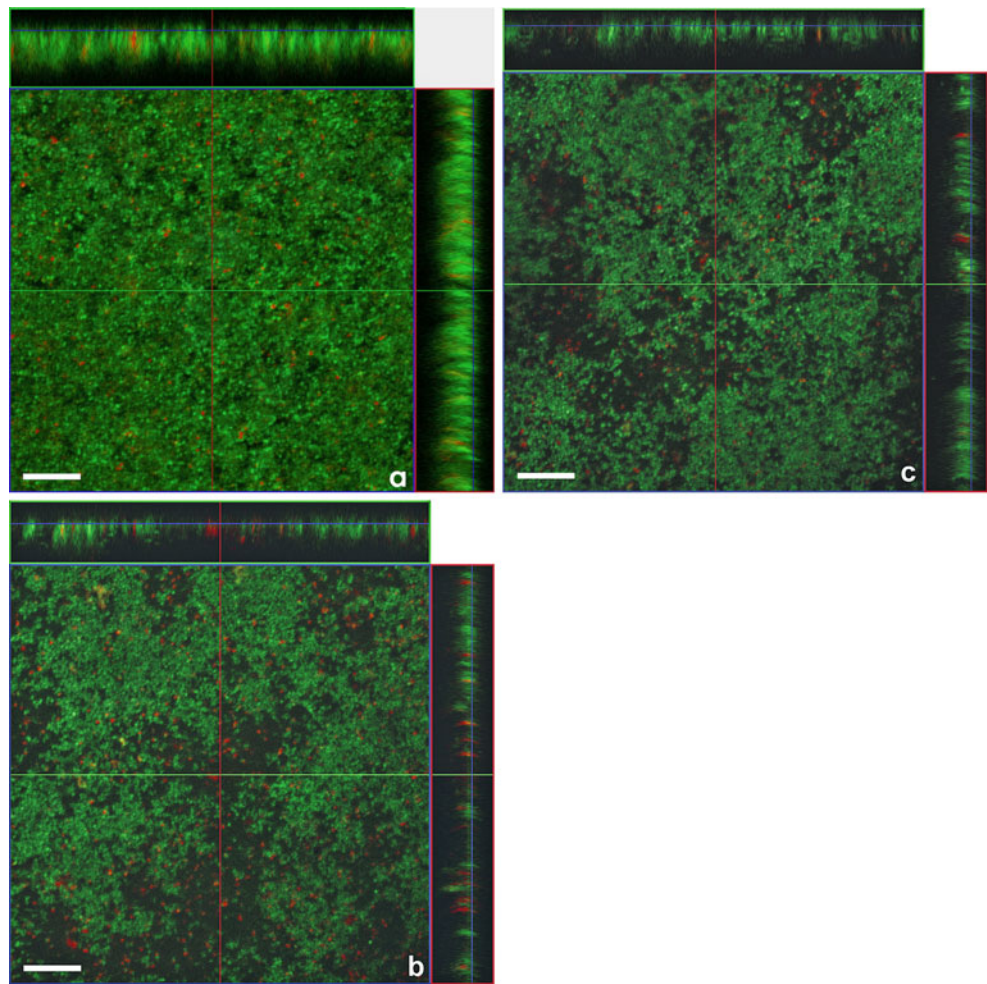
**Detection of slime production by Congo red agar plate test and biofilm assay** The ability to form slime was tested on Congo red agar (Arciola et al. 2002). Briefly, isolates were incubated on brain heart infusion agar (Oxoid) supplemented with 0.8  $\mu$ g/mL of Congo red (Sigma) and 36  $\mu$ g/mL of sucrose. The plates were subsequently incubated for 24 h at 37 °C and additionally overnight at room temperature. Slime-producing strains develop black colonies, whereas slime non-producing isolates form red colonies. Biofilm formation of *S. aureus* cell on polystyrene was quantified by using the microtiter plate assay (Kim et al. 2008; Fredheim et al. 2009). Isolates were cultivated overnight in 96-well flat-bottomed tissue culture plates at 37 °C with trypticase soy broth (bioMérieux, France) supplemented with 0.25 % glucose as a growth medium. After incubation, the cultures were gently removed, the wells were washed three times with phosphate-buffered saline, and air dried and stained with 0.4 % crystal violet solution for 10 min. The plate was washed, the adherent cells were resuspended in ethanol–acetone mixture (70:30), and finally the absorbance at 490 nm was determined. Isolates were considered biofilm-positive if they had an  $A_{490} > 0.25$ . Each isolate was tested in triplicate.

**Confocal laser scanning microscopy** Overnight cultures of different strains were added to cell culture chamber wells 9Lab-TekII, (Nalge Nunc International) and statically incubated for 24 h as described by Qin et al. (2007). Biofilms and bacteria were stained by using SYTO 9 and propidium iodide (PI) (Live/Dead BacLight Bacterial Viability kits, Invitrogen) for 15 min and observed under the microscope (Carl Zeiss LSM 510-Axiovert 200 M) equipped with detector and filter sets for monitoring SYTO9 and PI. The Carl Zeiss confocal software was used for the analysis of biofilm images, which allowed for collections of stacks three-dimensional reconstruction.

**Table 1** Distribution of *clfA*, *clfB*, *fib*, *fnbA*, *fnbB*, *can*, *eno*, *ebpS*, *bbp*, and *ica* genes in MRSA isolates

Type of infection	No. of strains	No. (%) of <i>clfA</i> -positive	No. (%) of <i>clfB</i> -positive	No. (%) of <i>fib</i> -positive	No. (%) of <i>fnbA</i> -positive	No. (%) of <i>fnbB</i> -positive	No. (%) of <i>can</i> -positive	No. (%) of <i>eno</i> -positive	No. (%) of <i>ebpS</i> -positive	No. (%) of <i>bbp</i> -positive	No. (%) of <i>ica</i> -positive
Pneumonia	29	28 (97)	28 (97)	21 (72)	0 (0)	15 (51)	11(38)	21 (72)	3(10)	0 (0)	20 (69)
Bacteremia	14	14 (100)	14 (100)	10 (71)	0 (0)	8 (57)	7 (50)	9 (64)	3 (21)	0 (0)	9 (64)
Wounds	16	16 (100)	16 (100)	11 (69)	0 (0)	8 (50)	10 (62)	15 (94)	4 (25)	0 (0)	13 (81)
Skin and soft tissue infections	11	10 (91)	11 (100)	7 (63)	0 (0)	5 (45)	6 (54)	7 (63)	0 (0)	0 (0)	8 (72)
Otitis	4	4 (100)	4 (100)	4 (100)	0 (0)	1 (25)	0 (0)	3 (75)	0 (0)	0 (0)	3 (75)
Ophthalmia	1	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Urinary tract infections	3	3 (100)	3 (100)	2 (67)	0 (0)	1 (33)	1 (33)	2 (67)	1 (33)	0 (0)	1 (33)
Arthritis	2	2 (100)	2 (100)	1 (50)	0 (0)	1 (50)	1 (50)	0 (0)	1 (50)	0 (0)	2 (100)

**Fig. 1** CLSM images of 24-h biofilms of *S. aureus* MPU S 8 (a), MPU S 6 (b), and MPU S 37 (c) stained with SYTO9 (green cells) and PI (red cells). The *ica*-positive strain MPU S 8 showed compact structure. The *ica*-negative/FNBP-positive strains (MPU S 6) and *ica*-negative/FNBP-negative strain (MPU S 37) showed less condensed biofilm structures than MPU S 8 strain. The common characteristic of biofilm produced by the *S. aureus* strains was the low number of dead cells (red cells) observed in mature biofilms. Bar=20  $\mu$ m



**Detection of adhesins and biofilm-encoding genes** Isolation of DNA was performed by using the Genomic DNA Plus kit (A&A Biotechnology, Poland). A multiplex polymerase chain reaction (PCR) was applied for simultaneous amplification of the *bbp*, *can*, *eno*, *ebps*, *fnbB*, *fnbA*, *fib*, *clfA*, and *clfB* genes (Tristan et al. 2003). Another PCR assay was applied to determine the presence of the *icaA* and *bap* genes (Cucarella et al. 2001; Peacock et al. 2002). The amplification products were electrophoresed in 1.5 % agarose gel, stained with ethidium bromide, visualized on a UV light transilluminator, and documented with V.99 Bio-Print system (Vilber Lourmat, Torcy, France).

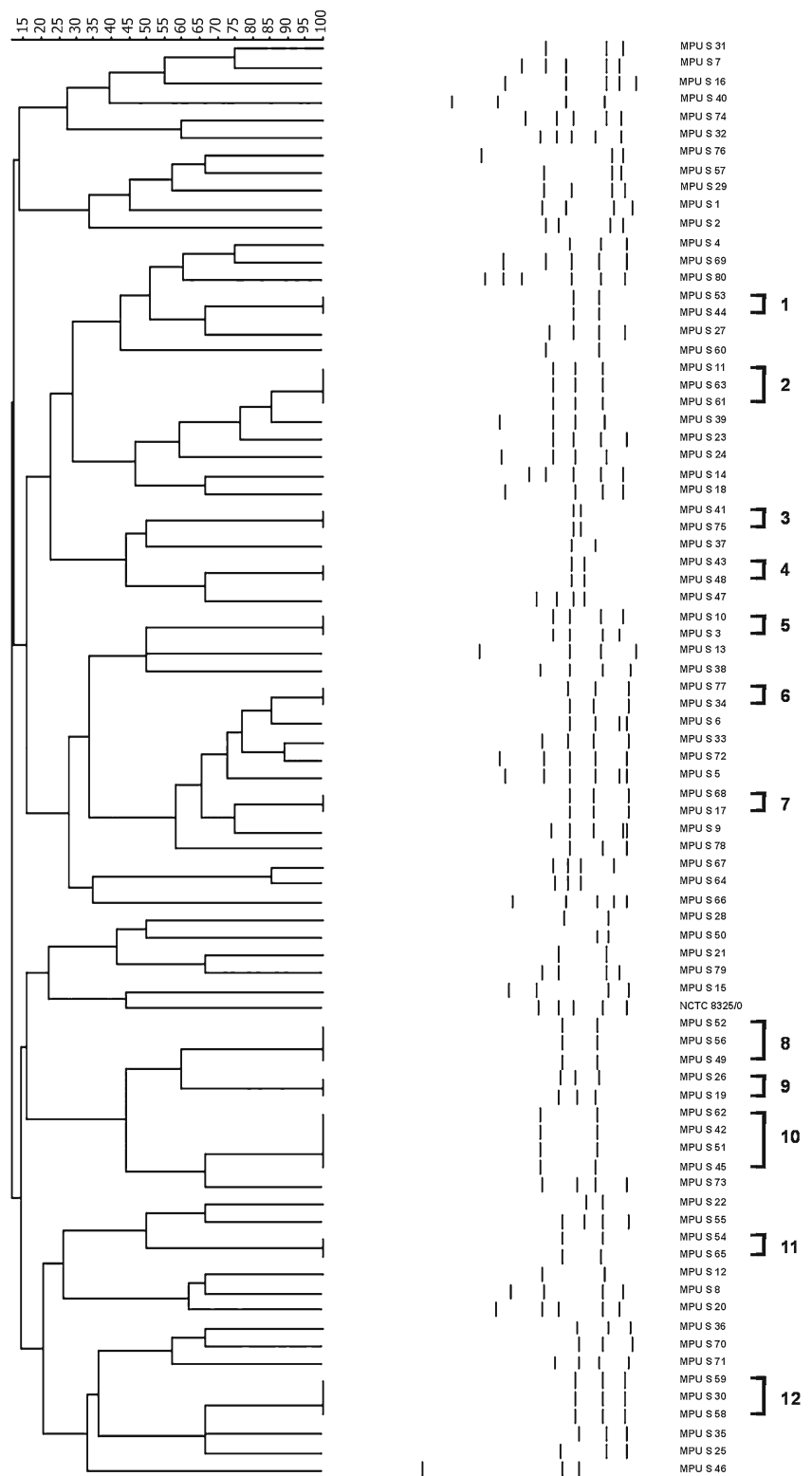
**Multiple-locus variable-number tandem repeat analysis** A PCR was applied to simultaneously amplify part of the hyper-variable number of tandem repeat (VNTR) regions of the *spa*, *sspsA*, *clfA*, *clfB*, *sdrC*, *sdrD*, and *sdrE* genes (Sabat et al. 2003). GelCompar II (version 3.5; Applied Maths, Kortrijk, Belgium) software was used to analyze the MLVA banding patterns. The Dice band-based similarity coefficient was calculated with band position tolerance of 1 %. MLVA dendrogram was constructed by unweighted pair-group method

(UPGMA) with arithmetic means. We defined clusters as two or more isolates with more than 95 % similarity.

## Results and discussion

Our results indicated that the vast majority (93 %) of *S. aureus* isolates had the ability to produce biofilm in vitro. Of the 74 biofilm-positive strains, 56 carried the *icaA* (76 %) gene and produced slime on Congo red agar (CRA). Arciola et al. (2001) have reported that only 61 % of *S. aureus* strains from catheter-associated infections carry *icaA* genes and produce slime on CRA. Data reported by others have indicated that nearly all *S. aureus* strains carry *ica* genes (Peacock et al. 2002; Rodhe et al. 2007). Our results indicated that two *icaA*-positive strains did not have the ability to produce slime and form biofilm. It could be explained by appearance of rare slime-negative mutants, which emerged as a result of passages and prolonged culture on CRA plate (Arciola et al. 2001). It is also well-known that the *ica* expression is strongly influenced by environmental factors such as glucose, temperature, osmolarity, and growth in anaerobic conditions (Beenken et al.

**Fig. 2** Dendrogram generated from MLVA profiles of *S. aureus* strains by the UPGMA with arithmetic means. The Dice band-based similarity coefficient was calculated with a band position tolerance of 1 %. The scale bar represents similarity



2004; Kim et al. 2008). None of the MRSA strains were positive for *bap* genes. We found that 23 % of MRSA strains were biofilm-positive although they did not carry *icaA* and *bap* genes. These data provided evidence for *ica*-independent biofilm formation in MRSA strains. Our results are coincident

with the findings reported by O'Neill et al. (2007, 2008) and Izano et al. (2008). It should be noted that 50 % of MRSA strains carry multifunctional surface proteins fibronectin-binding protein B (FNBP). According to O'Neill et al. (2008), FNBP proteins are involved in intercellular accumu-

lation and biofilm development in *S. aureus* strains. Importantly, 9 of 18 *ica*-negative and biofilm-positive strains carry *fnbB* genes, whereas 9 of 18 *ica*-negative and biofilm-positive strains did not carry *fnbB* genes. The absence of PIA, BAP, and FNBP among these nine biofilm-positive strains strongly suggests that *S. aureus* may use also different strategies to form biofilm. It has been indicated that DNA is a structural component of the *S. aureus* matrix and extracellular DNA fragments of >11 kb can function as intercellular adhesins (Izano et al. 2008). Furthermore, wall teichoic acids have been shown to participate in biofilm cohesion (Gross et al. 2001). We compared the architecture of biofilm created by *S. aureus* strains by using confocal laser scanning microscopy (CLSM). Six biofilm-positive *S. aureus* strains were selected for this study: two strains (Bacteriology Collection of Department of Microbiology A. Mickiewicz University, Poznań (MPU S) 8 and 14) positive for *ica* genes, two (MPU S 6 and 13) positive for *fnbB*, and two (MPU S 23 and 37) negative for *ica* genes and *fnbB* gene. We analyzed the entire thickness of bacterial biofilms as well as the surface of chamber well coverage of biofilms. Figure 1 exemplifies the differences between the *ica*-positive (Fig. 1a) and *ica*-negative strains (Fig. 1b, c). Microscopic images showed that the two *ica*-positive strains created the thickest biofilms with compact architecture. The thickness of biofilm formed by MPU S 8 and 14 strains was about 25 and 23  $\mu\text{m}$ , respectively. In contrast, biofilm created by *ica*-negative strains was much thinner than that of *ica*-positive strains. Four *S. aureus* strains (MPU S 6, 13, 23, and 37) formed relatively thin biofilm structures; the average thickness of biofilm formed by *ica*-negative strains was 13  $\mu\text{m}$ . Beside the differences in biofilm thicknesses, we also observed the differences in the area covered by biofilm. The CLSM images of MPU S 6, 13, 23, and 37 *S. aureus* biofilms showed that not all chamber wells were covered by multiple layers of cells. The structures of these biofilms were relatively loose and there were areas on chamber wells with only loosely attached cells or cellular aggregates. It should be emphasized that *S. aureus* MPU S 8 and 14 formed biofilm structures in which bacterial cells were very closely packed. The common characteristic of biofilm produced by the *S. aureus* strains was the low number of dead cells observed in mature biofilms (Fig. 1). However, we observed more dead cells in the *ica*-negative strains.

The vast majority of the isolates were positive for genes encoding fibrinogen-binding protein (ClfA, ClfB, and Fib) and *eno* gene encodes laminin-binding protein. The genes encoding fibronectin-binding protein A (*fnbA*) were absent in all isolates, whereas *fnbB* genes encoding fibronectin-binding protein B (*fnbB*) were present in 50 % of the strains. None of the *S. aureus* strains from our collection carried genes encoding bone sialoprotein-binding protein. The lack of *bbp* genes in *S. aureus* isolated from urinary tract infections has been also reported by Baba-Moussa et al. (2008). Rodhe et al. (2007) have observed high prevalence of *bbp*

genes in *S. aureus* strains isolated from prosthetic hip and knee joint infections. This discrepancy in the results may be related to the site of infection from which the strains were isolated. The gene encoding the elastin-binding protein (*ebpS*) was present only in 15 % of the MRSA, whereas the gene encoding collagen-binding protein was present in 45 % of *S. aureus* strains.

In this study, we used MLVA analysis to evaluate the clonal structure of *S. aureus* strains. It has been found that MLVA method is very useful to determine clonality and the spread of *S. aureus* strains in hospitals in short-time epidemiological investigation (Łuczak-Kadlubowska et al. 2008). On the basis of MLVA profiles, we identified 12 clusters at 100 % similarity level (Fig. 2). As shown in the dendrogram, the largest cluster comprised four isolates. The remaining clusters consisted of two to three strains. The fact that 12 clusters were identified next to 52 unique genotypes indicates a large genetic diversity among *S. aureus* strains. We compared the presence of *icaA*, *fib*, *fnbB*, *cna*, *eno*, and *ebpS* within each of the 12 clones. The *clfA*, *clfB*, *fnbA*, *bbp*, and *bap* genes were excluded from this comparison, as they were present or absent in almost all isolates. Seven clusters included both *ica*-positive and *ica*-negative strains, whereas five clusters included only *ica*-positive strains. Ten clusters contained isolates that carried different genes (*fib*, *fnbB*, *cna*, *eno*, and *ebpS*) mediating adhesion to host matrix proteins. Hence, the distribution of individual genes was not influenced by the clonality of the population. Taken these results together, we did not find a specific clone occurring preferentially in the hospital environment. There was also evidence that most MRSA strains were able to produce slime and form biofilm in *ica*-dependent mechanisms. On the other hand, we found some clinical strains that produced biofilm in *ica*-independent mechanisms.

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