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*Full Length Research Paper*

# Analysis of biofilm formation and associated gene detection in *Staphylococcus* isolates from bovine mastitis

**Li Li[1,2], Hong-jun Yang[1]*, Dai -cheng Liu[2], Hong-bin He[1], Chang-fa Wang[1], Ji-feng Zhong[1], Yun-dong Gao[1] and Yanjun Zeng[3]***

[1]Milch Cow Research Center of Agricultural Science Academy of Shandong Province, Jinan, Shandong 250100, P. R. China.
[2]College of Life Science, Shandong Normal University, Jinan, Shandong 250014, P. R. China.
[3]Beijing University of Technology, 100 Pinglyuan, Chaoyang District, Beijing, 100124, P. R. China.

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**The objective of this study was to investigate the biofilm-forming ability and distribution of biofilm associated genes in clinically isolated *Staphylococcus* in bovine mastitis. Silver staining, scanning electron microscopy (SEM) and crystal violet staining were conducted for the detection of biofilm-forming ability in 24-well plates. The *bap*, *icaAD*, *icaBC*, *Staphylococcal* accessory regulator (*sar*), accessory gene regulator (*agr*), *sigB*, clumping factor A (*clfA*), *clfB*, fibronectin-binding proteins (*fnbpA*) and *fnbpB* genes were amplified by polymerase chain reaction (PCR). Formation of biofilms was found macroscopically in 120 of the 137 strains after being stained with silver (biofilm-forming rate 87.6%). Five strains did not adhere to the surface of the silica gel after being stained with crystal violet, while the remaining 132 strains did adhere. *Bap* was amplified in 57 isolates, and *icaAD* and *icaBC* were isolated in 43 and 54 strains, respectively. *SigB*, *sar* and *agr* were amplified in 73, 49 and 38 isolates, respectively, and *clfA* and *clfB* were isolated in 76 and 50 strains, respectively. *FnbpA* was present in 52 strains and *fnbpB* in 26 isolates. Our study reveals that *bap*, *sigB*, *sar*, *icaAD* and *icaBC* may be crucial biofilm associated genes since these genes were present more often in biofilm-positive strains than in biofilm-negative strains. There was no obvious difference between the frequencies of *agr* in the biofilm-positive strains and biofilm-negative strains, which indicates that the role of agr in biofilm development is still controversial. The distribution of *clfA*, *clfB*, *fnbpA* and *fnbpB* in biofilm-positive strains were not greatly different from that in biofilm-negative strains.**

**Key words:** Bovine mastitis *Staphylococcus*, biofilm, silver staining, crystal violet staining.

## INTRODUCTION

*Staphylococcus*, with several characteristic poly-saccharides and adhesion protein factors on the surface, is likely to form a bacterial biofilm. Biofilms are related to pathogenicity and it has been proposed that *Staphylococcus* biofilms are major causes of recurrent and chronic mastitis in dairy cattle (Melchior et al., 2007). Studies on the ability of *Staphylococcus* to form biofilms and the underlying mechanisms may provide new ideas for the prevention and treatment of bovine mastitis.

*Staphylococcus* biofilm formation mechanisms are complex and include the participation of many kinds of proteins. The biofilm-associated protein (*bap;* 254 KD*)* positive *Staphylococcus* strains show a high capacity to infect and persist in the mammary gland, which is closely related to the biofilm formation of gram-positive bacterium (Cucarella et al., 2004). Bap, which is a member of the *Staphylococcus* biofilm related protein family, plays an important role in the process of biofilm formation, helping neighboring bacteria to form biofilms, and is related to pathogenic infection (Lasa et al., 2006). Furthermore, the biofilm forming ability is lost after *bap* gene knockout

*Corresponding author. E-mail: longfei1997@sina.com, yjzeng@bjut.edu.cn.

(Chen, 2009).

In the process of biofilm formation, the accumulation and development of a mature stage mainly depends on the polysaccharide adhesions that promote bacterial accumulation, especially polysaccharide intercellular adhesion (PIA). The *icaADBC* operon participates in biofilm formation by encoding enzymes involved in the synthesis of PIA, with regulation by *Staphylococcal* accessory regulator (*sar*), accessory gene regulator (*agr*) and *sigB* (Frank and Patel, 2007). The clumping factor (Clfa) and fibronectin-binding proteins (Fnbp) mediate bacterial adherence, however, the question of whether they play an influential role in biofilm formation remains unanswered.

Costerton et al. (1999) initiated research on biofilms in 1978 and presented a theory. Since then, researchers have been focusing on the mechanisms of *Staphylococcus* biofilm formation and most have used standard strains instead of clinical isolates. In China, research on biofilms only began recently and the research has mainly concentrated on *Staphylococcus* clinical isolates from humans. The aim of the present study was to test the biofilm-forming capability of bovine mastitis *Staphylococcus* strains and to analyze the distribution of biofilm-associated genes.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

The 137 *Staphylococcus* strains used in this study were isolated from the milk of bovines with mastitis in Shandong province. The bacteria were identified and preserved in our laboratory. Bacteria were stored at -70°C and were freshly cultivated in Luria-Bertani medium before the experiment.

### Silicone elastomer slice biofilm formation assays and silver staining

Silicone elastomer slices (1 × 1 $cm^2$) were cut from 1 mm-thick medical grade silicone elastomer sheetings. Biofilm formation was conducted using a modified plate assay. One milliliter of sterile ticase soy broth (TSB) and 10 µl of an overnight cultured *Staphylococcus* strain were added to each well of a 24-well plate. Slices were placed in the bottom of the wells with sterile forceps and were incubated at 37°C in a biochemical incubator for seven days. The medium was changed every two days. After incubation, the culture medium was discarded and 1 ml of sterile phosphate buffered saline (PBS) was added to each well three times to remove planktonic bacteria. Each silicone elastomer slice was divided into two, one for silver staining and the other for scanning electron microscopy (SEM). Each slice was observed through an optical microscope after silver staining. Slices in wells containing uninoculated medium served as negative controls and biofilm qualitative results obtained through SEM served as positive controls (Li et al., 2003).

### Crystal violet staining

Slices were incubated as described above for biofilm formation, and 1 ml of sterile 0.9% NaCl solution was added three times to remove planktonic bacteria and impurities. 200 µl of methanol was added to fix the biofilm and was then discarded 15 min later. After being air dried, biofilms were stained with 200 µl 2% crystal violet for 5 min, rinsed under running water to remove excess stain, and air dried over night. Then stained slices were destained with 33% glacial acetic acid and analyzed by reading the optical density at 570 nm ($OD_{570}$). Uninoculated slices were stained as blank controls.

The OD value can reflect the degree of biofilm adhesion to contact surfaces. Based on a critical OD value ($OD_c$ is equal to the mean value of blanks and three times its standard deviation), the biofilm can be classified into the following categories: when $OD \leq OD_c$, the biofilm did not adhere to the contact surface, which was recorded as (0); when $OD_C < OD \leq 2OD_c$, the biofilm adhered to the contact surface weakly, which was recorded as (+); when $2OD_C < OD \leq 4OD_c$, the adhesion between the biofilm and the contact surface was moderate, which was recorded as (++); and when $OD > 4OD_c$, adhesion between the biofilm and contact surface was strong, which was recorded as (+++) (Duan et al., 2008).

### DNA extraction

Genomic DNA was prepared as follows: 5 µl of bacteria were inoculated in 5 ml LB culture medium, which was then cultured at 37°C for 16 h with shaking. 1.5 ml bacteria were placed into a 1.5 ml centrifuge tube and centrifuged at 12000 r/min for 3 min. The supernatant was discarded and the precipitation was suspended with 1,1,1-trinitroethane (TNE) and then centrifuged as above. The supernatant was discarded again and the precipitation was resuspended in 200 µl Tris-EDTA (TE). Subsequently, 4 µl of lysozymes was added and fully mixed to inoculate at 37°C. After 1 h, 4 µl of proteinase K was added and the reaction occurred for 1 h at 55°C. After cooling to room temperature, it was boiled for 10 min and then placed in ice for 3 min immediately. Finally, it was centrifuged for 5 min at 12000 r/min and the supernatant was stored in a -20°C refrigerator.

### Analysis of genes involved in biofilm formation using a polymerase chain reaction (PCR) assay

Using the bacterial chromosomes prepared above as templates, the thermal cycling procedure consisted of a predenaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, annealing (annealing temperatures are presented in Table 1) for 20 s, and extension at 72°C for 50 s, with a final elongation step at 72°C for 5 min. The sizes of the PCR products were analyzed by electrophoresis on 1% (wt/vol) agarose gels. The primers used in the PCR assays, as well as the expected amplified product sizes and the references are presented in Table 1.

## RESULTS

### Qualitative detection of biofilm formation

After silver staining, the biofilm condition was observed by an ordinary optical microscope. There is a piece of film-like black floc on the silicone elastomer slices if a biofilm was formed, whereas there are dark specks or almost nothing in the control group (Figure 1). Biofilms were found macroscopically in 120 of the 137 strains after silver staining, and the biofilm formation rate was 87.6%. SEM visualization showed that cells were growing in communities and organized into a three-dimensional

**Table 1.** Primers used in the present study.

| Target gene | Primer sequence | Annealing temperature (℃) | Product size (bp) | Reference |
|---|---|---|---|---|
| *bap* | 5'-CCCTATATCGAAGGTGTAGAATTG-3'<br>5'-GCTGTTGAAGTTAATACTGTACCTGC-3' | 62 | 971 | Cucarella et al. (2001) |
| *icaAD* | 5'-CCTAACTAACGAAAGGTAGG-3'<br>5'-TTAGCGTTGGGTATTCCCTC-3' | 55 | 1266 | Sun et al. (2009) |
| *icaBC* | 5'-ATGGTCAAGCCCAGACAGAG-3'<br>5'-GCACGTAAATATACGAGTTA-3' | 55 | 1188 | Sun et al. (2009) |
| *sar* | 5'-CGGTACCGTTGATTTGGGTAGTATGC-3'<br>5'-TTGCCATGGTTAAAACCTCCC-3' | 55 | 867 | Sun et al. (2009) |
| *agr* | 5'-GTGCCATGGGAAATCACTCCTTCC-3'<br>5'-TGGTACCTCAACTTCATCCATTATG-3' | 55 | 976 | Sun et al. (2009) |
| *sigB* | 5'-CGGATCCGGTGTGACAATCAGTATGAC-3'<br>5'-CGGAATTCGCGACATTTATGTGGATACAC-3' | 55 | 937 | Sun et al. (2009) |
| *fnbpA* | 5'- CATAAATTGGGAGCAGCATCA-3'<br>5'- ATCAGCAGCTGAATTCCCATT-3' | 55 | 127 | Vancraeynest et al. (2004) |
| *fnbpB* | 5'- GTAACAGCTAATGGTCGAATTGATACT-3'<br>5'- CAAGTTCGATAGGAGTACTATGTTC-3' | 55 | 524 | Tristan et al. (2003) |
| *clfA* | 5'- ATTGGCGTGGCTTCAGTGCT-3'<br>5'- CGTTTCTTCCGTAGTTGCATTTG-3' | 55 | 292 | Primer 5.0 designed |
| *clfB* | 5'- ACATCAGTAATAGTAGGGGGCAAC-3'<br>5'- TTCGCACTGTTTGTGTTTGCAC-3' | 55 | 205 | Primer 5.0 designed |

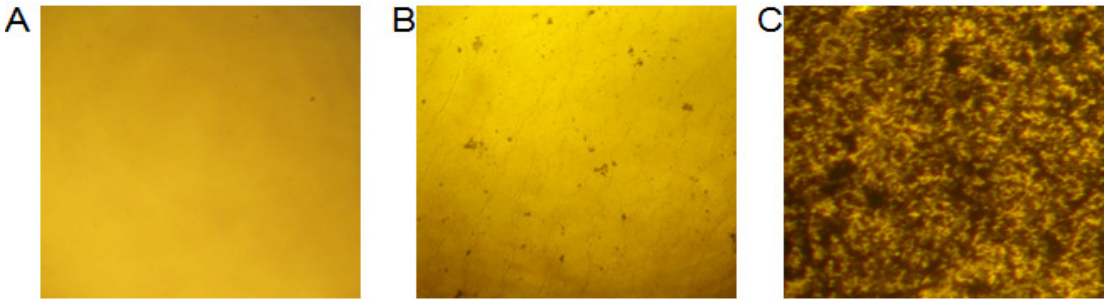


**Figure 1.** Optical micrographs of *Staphylococcus* biofilms by silver stainning (100 X): (A) a blank control; (B) a biofilm-negative strain; (C) a biofilm-positive strain.

architecture (Figure 2A). Figure 2B shows a scanning electron micrograph of one biofilm-negative strain in which cells were growing intermittently or developed into microcolonies.

## Quantification of biofilm formation

Bacterial biofilms were quantified by $OD_{570}$, five of the 137 strains did not adhere to the surface of a silica gel, 75 strains adhered to the contact surface weakly, 55 strains adhered moderately and two strains adhered strongly (Table 2).

## Genes involved in biofilm formation

57 out of the 137 isolates included in this study were positive for *bap*. *IcaAD* and *icaBC* were amplified in 43 and 54 strains, respectively. 73 isolates harbored *sigB*, 49 isolates harboured *sar* and 38 isolates harboured *agr*. *clfA* and *clfB* were amplified in 76 and 50 strains, respectively. 52 strains carried *fnbpA* and 26 strains were positive for *fnbpB* (Table 3). Assays were repeated two

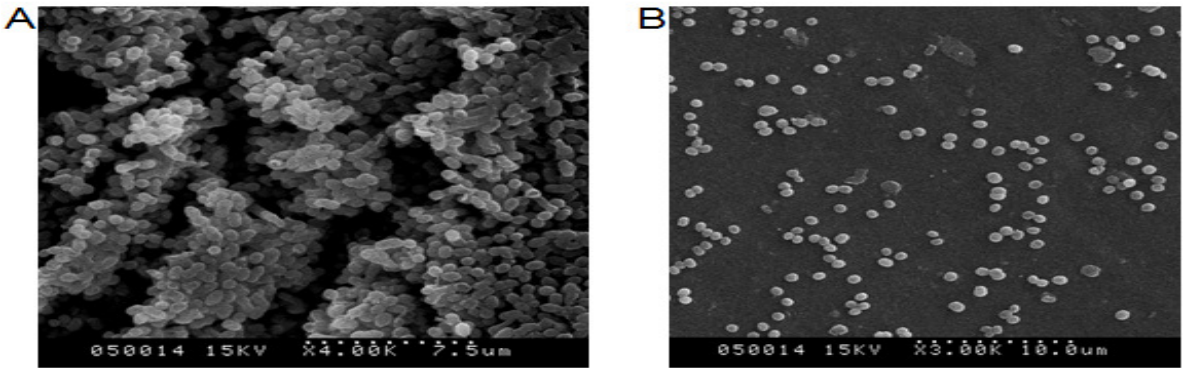


**Figure 2.** Scanning electron micrograph of *Staphylococcus* biofilms: (A) A biofilm-positive strain (x4.00 K); (B) a biofilm -negative strain (×3.00 K).

**Table 2.** Quantitative detection results of bacterial biofilms.

| Degree of biofilms adhesion to contact surface | Number |
|---|---|
| No adhesión (0) | 5 |
| Weak adhesion (+) | 75 |
| Moderate adhesión (++) | 55 |
| Strong adhesión (+++) | 2 |

times.

## DISCUSSION

Bacteria are attached to non-active objects or living surfaces aggregate in a hydrated polymeric matrix self-synthesized to form biofilms (Costerton et al., 1999). Biofilm associated genes must be studied in order to investigate the molecular foundation of *Staphylococcus* variation and the pathogenic mechanism of chronic infection caused by *Staphylococcus*. Bap-positive isolates are able to infect and persist in the bovine mammary gland and are less susceptible to antibiotics when forming biofilms *in vitro* (Cucarella et al., 2004). Nevertheless, a collection of 262 isolates that were obtained from other species and from various locations were tested with PCR using published primers and dot-blots. The results indicate that none of the isolates carried the *bap* gene (Vautor et al., 2008). In this study, the *bap* gene was amplified in 51 of 116 biofilm-positive strains, indicating that the *bap* gene was present in, not all, but some of the biofilm-positive strains. Six strains that carried the *bap* gene could generate biofilms, which is inconsistent with previous reports.

*Ica* is commonly found in *Staphylococcus* (Fowler et al., 2001) and is directly related to the formation of bacterial biofilms as an essential factor. The *ica* operon consists of four genes (*icaA*, *icaD*, *icaB* and *icaC*) and their coding product co-synthesizes the key material, PIA, for *Staphylococcus* adhesion in the process of biofilm formation. In this study, there were only 41 strains that carried *icaAD* and 51 strains carried *icaBC* in 116 biofilm-positive strains. Furthermore, a few isolates that carried *ica* could not form a biofilm, likely because *ica* expression is regulated by multiple accessory regulators, or it may be due to the existence of other *ica*-independent biofilm formation mechanisms. In addition, strains that carried *icaAD* or *icaBC* alone were discovered in this study, which was inconsistent with previous reports. This may be due to the differences between human and bovine *Staphylococcus* strains.

*Ica* expression is regulated by multiple genes such as *sigB*, *Staphylococcal* accessory regulator (sar) and accessory gene regulator (*agr*). They may interact with each other and regulate biofilm formation through *ica* operon expression eventually. *SigB*, *sar* and *agr* have been shown to perform important functions in *Staphylococcus* life processes. *Sar*, a *Staphylococcal* accessory regulator, widely regulates intracellular and extracellular protein expression. Mutations show a significant decrease of *ica* operon expression and subsequently affect the synthesis of PIA (Valle et al., 2003). *Agr*, an assessory gene regulater of *Staphylococcus aureus*, controls the expression of a series of toxins and virulence factors and interaction with the innate immune system (Kong et al., 2006). However, the role of *agr* in the infection process seems to be controversial (Kong et al., 2006). In biofilm-related infections, its role is to reduce rather than induce biofilm formation and virulence factor expression. *Agr* increased expression of surface-like peptides leading to detachment of biofilms (Yao et al., 2005); that is, cells dislodged from the biofilm structure as planktonic cells disseminate to a distant site to reform another biofilm. *SigB* majorly regulates the expression and transcription of bacterial genes under stress conditions. Its level and activity adjust to environmental pressure (Rachid et al., 2000). In this study, *sigB* was amplified in 73 strains, *sar* in 49 strains and *agr* in 38 strains. In contrast to previous reports, not all the biofilm-positive strains carried *sigB*, *sar* and *agr*, and these genes were also amplified in a small amount of

**Table 3.** Results of biofilm-associated gene testing.

| Gene | Number of (+) isolates in biofilm-positive strain | | Number of (-) isolates in biofilm-negative strain | | Total number of (+) isolate | |
|---|---|---|---|---|---|---|
| | (n) | % | (n) | % | (n) | % |
| *bap* | 51/116 | 44.0 | 6/21 | 28.6 | 57/137 | 41.6 |
| *sigB* | 65/116 | 56.0 | 8/21 | 38.1 | 73/137 | 62.9 |
| *sar* | 46/116 | 40.0 | 3/21 | 14.3 | 49/137 | 35.8 |
| *agr* | 34/116 | 29.3 | 4/21 | 19.0 | 38/137 | 27.7 |
| *icaAD* | 41/116 | 35.3 | 2/21 | 9.5 | 43/137 | 31.4 |
| *icaBC* | 51/116 | 44.0 | 3/21 | 14.3 | 54/137 | 39.4 |
| *clfaA* | 67/116 | 57.8 | 9/21 | 42.9 | 76/137 | 55.5 |
| *clfaB* | 44/116 | 37.9 | 6/21 | 28.6 | 50/137 | 34.5 |
| *fnbpA* | 42/116 | 36.2 | 10/21 | 47.6 | 52/137 | 38.0 |
| *fnbpB* | 24/116 | 20.7 | 2/21 | 9.5 | 26/137 | 19.0 |

biofilm-negative strains.

Initial adhesion stages of *Staphylococcus* are involved with a variety of surface proteins and adhesion factors that mediate *Staphylococcus* adhesion to the host cells. This is the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family, which includes fibronectin-binding proteins A (FnBPA), fibronectin-binding proteins B (FnBPB), collagen-binding protein (Can), fibrinogen-binding protein (Fbe), clumping factor A (ClfA) and clumping factor B (ClfB). The MSCRAMM family is important for adhesion between *Staphylococcus* and polymers *in vitro*; e.g., *clfA* mediates bacterial attachment to plasma clots formed *in vitro* and to plastic biomaterial (Foster et al., 1998). In this study, the distribution of *clfA*, *clfB* and *fnbpB* in biofilm-positive strains is not different from that in biofilm-negative strains; the former is about 10% more than the latter. When it comes to *fnbpA*, the former is 11.4% lesser than the latter.

In the present study, silver staining and crystal violet staining were used for qualitative and quantitative detection of the capacity for biofilm formation by *Staphylococcus* in dairy cattle mastitis. A combination of the two methods is more effective for detection of biofilm formation. Biofilm forming ability and the related genes of clinical *Staphylococcus* isolates from mastitis cattle were detected. The gene testing results revealed that *bap*, *sigB*, *sar*, *icaAD* and *icaBC* were significantly more frequent in biofilm-positive strains than in biofilm-negative strains. There was no significant difference between the frequency of *agr* in biofilm-positive strains as compared to biofilm-negative strains and, therefore, the role of *agr* in biofilm development is still controversial. Also, the distributions of *clfA*, *clfB*, *fnbpA* and *fnbpB* in biofilm-positive strains were not different as compared to biofilm-negative strains.

*Staphylococcus* biofilm formation is the complex result of multiple gene control. Understanding the role of each gene in biofilm formation and how they are modulated will provide an important basis for the control of *Staphylococcus* biofilm formation. Our results may contribute to further study of *Staphylococcus* biofilm formation mechanisms and the prevention or treatment of bovine mastitis. Furthermore, gene knockout studies should be carried out as well as antibody growth inhibition assays of biofilms to verify the associated genes after their expression in prokaryotic cells.

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