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BIOFILM CHARACTERIZATION AND QUORUM QUENCHING IN PATHOGENIC STRAINS *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

A strategy for combating biofilm based bacterial infections different from antibiotic therapy is prevention of biofilm formation by quorum quenching and genetically modifying the pathogenic strains to reduce the pathogenic potential. Pathogenic strains of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia, Serratia marcescens* were screened for biofilm production by qualitative (TM, CRA, GCR), quantitative method (TCP) as well by inverted microscopy. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were found to be potent biofilm producers. The biofilms from these selected strains were then investigated for quorum quenching effects and natural genetic transformation. In the present study *Bacillus. subtilis* at 1:40 and 1:20 ratio in mixed culture with both *Staphylococcus aureus* and *Pseudomonas aeruginosa;* tannic acid at 100 mg/l and EDTA at 100 mM were determined to be effective quorum quenching agents. Natural genetic transformation in *Staphylococcus aureus* was investigated by using pBR322 plasmid DNA as donor. The transformation efficiency increased with increase in DNA concentration and was higher in case of young actively growing biofilms. We conclude that tannic acid a plant ingredient could be an effective nontoxic antibiofilm compound for strains *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Key words: Biofilm; *Staphylococcus aureus; Pseudomonas aeruginosa;* Quorum Sensing; Quorum Quenching; Natural Genetic Transformation

Abbreviations:

TCP	Tissue Culture Plate
TM	Tube Method
CRA	Congo Red Agar
GCR	Glass Coupon Reactor
EDTA	Ethylenediamine tetraacetic acid

Key words: Biofilms, *S. aureus*, *P. aeruginosa*, natural genetic transformation, Quorum sensing, Quorum Quenching

INTRODUCTION

Bacterial biofilm represents virulence factor which contributes to infections [6]. Biofilm is an assemblage of the microbial cells that is associated with a surface and usually enclosed in a matrix of polysaccharide materials. Biofilm is responsible for chronic bacterial infection, infection on medical devices and the contamination of food [18]. Biofilm producing *Staphylococci* frequently colonize catheters and medical devices and may cause foreign body related infections. They easily get attached to

polymer surfaces [11, 25]. Crampton et al. showed that like *Staphylococcus epidermidis*, *Staphylococcus aureus* also has *ica* gene locus encoding the function of intracellular adhesion and biofilm formation.

Each bacterial cell produces a basal level of signal molecules that can move in and out of cells via diffusion or active transport [21]. At low cell densities these pheromones are at low concentrations, and at high cell densities the signal molecules accumulate to a sufficient concentration and are involved in regulation of different biological functions, including production of virulence factors [21, 22]. *Pseudomonas aeruginosa* is an opportunistic pathogen, which mainly causes disease in individuals that are immunocompromised, have cystic fibrosis or suffer from serious burn wounds. It utilizes two *N*-acyl-homoserine lactone (AHL)-dependent quorum sensing systems termed *las* and *rhl*, which together regulate an extensive set of cell population density and growth phase-dependent virulence factors [4]. Further work by Dong et al. (2002) reported the enzymatic analysis with known *Bacillus* strains which indicated that all of the strains of *Bacillus thuringiensis* and the closely related species *Bacillus subtilis* and *Bacillus mycoides* tested produced AHL-inactivating enzyme. The AHL-dependent quorum sensing mechanisms determines virulence gene expression in pathogenic bacteria, hence could serve as a promising target for developing anti-biofilm approaches to control bacterial infections. There are some polyphenolic compounds having a gallic acid moiety (as for example, epigallocatechin gallate, ellagic acid as well as tannic acid) that are commonly produced by various plant species and has potential to specifically block AHL-mediated communication between bacteria [1] thereby preventing the biofilm formation. EDTA is a synthetic amino carboxylic chelating agent that forms strong complexes with cations that are required to stabilize the negatively charged polysaccharides holding the biofilm together. These findings could serve as basis to develop non-toxic and effective AHL antagonists for combating bacterial infections.

Natural genetic transformation is a process by which bacteria are able to take up and integrate exogenous free DNA from their environment. This process enables the recipient organisms to acquire novel genes or heritable traits, thereby promoting the emergence of genetic variation and the rapid evolution of virulence factors [19]. It may be possible to take advantage of natural transformation processes to modify the phenotypes of biofilm communities giving them specific and desirable functions. Studying genetic competence in biofilms is a prerequisite for understanding the mechanism of horizontal gene transfer occurring in natural environment [8]. Induction of genetic competence in mitis group of the genus *Streptococcus* is mediated by quorum sensing, which depends on a competence stimulating peptide (CSP) signalling system [14, 15, 17, 20]. Recent evidence suggests that the growth of bacteria in biofilms can facilitate horizontal gene transfer between bacterial species via either conjugation or transformation [6, 13, 26].

In the present study *S. aureus* and *P. aeruginosa* were found to be potent biofilm producers among the pathogenic strains screened for biofilm production (*S. aureus*, *P. aeruginosa*, *Klebsiella pneumonia, Serratia marcescens*, *Escherichia coli*) by qualitative, quantitative method (TCP) as well by inverted microscopy. The biofilms from these selected strains were then evaluated for quorum quenching effects and natural genetic transformation. To our knowledge this is the first report indicating natural genetic transformation in *S. aureus* biofilms. In our study *B. subtilis* culture supernatant, tannic acid and EDTA were examined for quorum quenching activity against *S. aureus* and *P. aeruginosa* biofilms.

Materials and Methods:

In our study we had used four different methods for biofilm production (TM, CRA, GCR and TCP). The pathogenic organisms *Staphylococcus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 424), *Klebsiella pneumonia* (MTCC 2696), *Serratia*

marcescens (MTCC 86) and *E.coli* (NCIM 2065) were screened for biofilm production.

In Qualitative Methods, *Tube method* was performed as described by Bose et al. (2009) in glass test tube using different media; micro-organisms were incubated for 72 hrs at 37°C in static incubator (Osworld laboratory incubator JRIC-9). Media was decanted after incubation and stained with 0.1% crystal violet. Formation of biofilm was indicated by ring at air-medium interface and button like structure at the bottom of the test tube. Tube method was also performed in plastic tubes incubated at 30°C for 72 hrs in 180 rpm shaker incubator (MIR-153; Sanyo Electric co. Ltd.)

Glass coupon reactor method was done using the method described by Charaf et al. (1999) on glass slides placed on tryptone soy agar incubated at 37°C for 72 hrs; stained with 0.1% crystal violet. Biofilm formation was indicated by patch formations on slide. Congo red agar method was also performed adapted by Bose et al. (2009). Micro-organisms were streaked on congo red agar plate containing brain heart infusion broth as a carbon source. Plates were incubated at 37°C for 24 hrs. Black colonies with a dry crystalline consistency indicated biofilm production.

In Quantitative Method, TCP was adapted from the procedure published by Bose et al. (2009). The optimal incubation period was 72 hrs for the biofilm formation. Experiment was performed in triplicates. The results were analysed by taking O.D at 570 nm wavelength with a micro ELISA autoreader (Thermo electron corporation; Multiskan ex). For data calculation, mean O.D values < 0.120 was considered nonbiofilm producer, 0.120 – 0.240 was moderate and > 0.240 was considered as strong biofilm producers. Microscopic images were taken by inverted microscope (Carl Zeiss Axiovert 40CFL) at 400x magnification.

In Qualitative Methods, Tube method was performed in glass test tube using tryptone soybroth media with 1% glucose; micro-organisms were incubated for 48 hrs at 37°C in static incubator. After 48 hrs incubation quorum quenching agents were added to the media and further overnight incubation was carried out at 37°C under static conditions. Media was decanted after incubation and stained with 0.1% crystal violet. Quorum quenching effect was observed in test compared to control. Tube method was also performed in plastic tubes incubated at 30°C for 48 hrs in 180 rpm shaker incubator.We have done in vivo quorum quenching by addition of *B. subtilis* culture to *S. aureus* and *P. aeruginosa* culture in ratio of 1:20 and 1: 40. In Quantitative Method, TCP was performed. Biofilm was produced in wells of ELISA micro titre plate. After 48 hrs incubation quorum quenching agents were added to the media and further overnight incubation was carried out at 37°C under static conditions. Media was decanted after incubation and stained with 0.1% crystal violet. Quorum quenching effect was observed in test compared to control. This experiment was performed thrice independently in triplicates. The results were analysed by taking O.D at 570nm wavelength with a micro ELISA auto reader. Natural Genetic Transformation experiment was performed using procedure described by Hendrickx et al. (2002). pBR322 plasmid isolated from *E. coli* DH5α cells was used for NGT studies on *S. aureus* at two different DNA concentration (0.5 µg/ml and 1.5 µg/ml) for two different biofilm maturation stages. Transformed colonies of *S. aureus* were screened on LB agar plate containing ampicillin and tetracycline antibiotics. Transformation efficiency was calculated by standard method.

Results and Discussion

Considering modified TCP as gold standard method compared to TM, CRA and glass coupon reactor (GCR) methods. True biofilm producers were positive by modified TCP, TM, GCR and CRA. False positive were biofilm producers by TM, GCR and CRA method but not by modified TCP method. False negatives were non-biofilm producers by TM and CRA methods but the same strains were biofilm producers by modified TCP method. True negatives were non-biofilm producers by all the methods. In our study *K. pneumonia* gave false

positive result and *P. aeruginosa* gave false negative by CRA method. *S. marcescens* was false positive by TM and GCR method (Table. 1).

Our studies revealed that *S. aureus* and *P. aeruginosa* were potent biofilm producers in SB when supplemented with 1% glucose and 2 % glucose, respectively (Fig. 1). These organisms also produced visible biofilm in glass and plastic tube method. TCP is better screening test for biofilm production than CRA, GCR and TM. The test is easy to perform and assess both qualitatively and quantitatively. PCR based methods are reported as indicators of virulence marker of staphylococcal infection [2, 3, 23]. The present study was aimed at optimizing the biofilm production for use in natural genetic transformation studies.

In quorum quenching studies *B. subtilis,* tannic acid and EDTA showed visible effect of quorum quenching and marked destruction of biofilm. *B. subtilis* showed increasing in-vivo quorum quenching effect at both 1:40 and 1:20 ratio in mixed cultures of *P. aeruginosa* and *S. aureus* indicating that AHL- lactonase produced by *B. subtilis* affects quorum sensing regulation. AHL lactonase hydrolyze the lactone ring in the homoserine moiety of AHLs, therefore it interferes with quorum sensing mechanism. Yi-Hu Dong et al. (2002) has identified the quorum quenching N-acyl homoserine lactonases from *B. thuringiensis* and closely related species. The applications of quorum quenchers in contolling bacterial infections are limited due to their toxicity. Tannic acid acts like a strong siderophore and chelate iron from the medium and make iron unavailable to microorganism thus causes the degradation of bacterial biofilm. Tannic acid was found to be a potent quorum quenching agent showing increasing biofilm inhibition at 50 mg/l and 100 mg/l for *P. aeruginosa* and *S. aureus*. The most effective quorum quenching agents against *S. aureus* biofilms was *B. subtilis* culture supernatant compared to 100 mM EDTA and 100 mg/l tannic acid (Fig. 2). The effective quorum quenching agents against *P. aeruginosa* biofilms was 100 mM EDTA followed by 100mg/l tannic acid and *B. subtilis* culture supernatant (Fig. 3). Microscopic images corresponding to the quorum quenching effects reveal destruction of biofilms mediated by *B. subtilis* culture supernatant as well as tannic acid and EDTA. Further studies are required to prove the invitro quorum quenching activity of purified AHL lactonase from *B. subtilis*. The blast analysis indicates 97% homology of *B. subtilis* AHL lactonase gene with that of *B. thuringiensis* gene. Ellagic acid present in many red fruits and berry was found to be ineffective in abolishing biofilm formation in *S. aureus* as reported by Durig et al. 2010. However we have observed that tannic acid could effectively prevent biofilm formation in *S. aureus* and being a plant ingredient should be non-toxic to humans.

The effect of DNA concentration and biofilm age on natural genetic transformation was investigated in biofilm of *S. aureus*. We have obtained transformation efficiency of $8.6 \pm 0.7 \times 10^3$ and $13.1 \pm 1.0 \times 10^3$ for 0.5 µg/ml and 1.5 µg/ml DNA respectively in one day biofilm. Subsequently transformation efficiency for 3 day biofilm with 0.5 µg/ml and 1.5 µg/ml was $1.6 \pm 0.6 \times 10^3$ and $11.3 \pm 1.4 \times 10^3$ respectively (Table. 2). The transformation efficiency increased with increase in the concentration of DNA. The biofilm age is an important factor in determining the competence of *S. aureus*. The transformation efficiency decreased with increase in biofilm age implying that competence was higher in one day old biofilm. One day biofilm could be considered as actively growing biofilm. Similar results with biofilm age and DNA concentration has been reported with natural genetic transformation in monoculture *Acinetobacter* sp. strain BD413 biofilms by Hendrickx et al. (2003). The induction of genetic competence requires a wide variety of conditions for different species and strains [14, 15]. In the three day biofilm the lower transformation efficiency was due to possibly formation of diffusion barrier caused by extracellular matrix as demonstrated by electron micrographs [19] or by cells that were less metabolically active or dead.

Higher concentration of DNA (10- 100 µg/ml) was not used in this study as it does not exist in natural conditions. Transformation was further confirmed by antibiotic susceptibility test on MHA plates (Fig. 4). pBR322 has been shown to

be homologous to many vector DNA hence was selected for transformation [24]. The *S. aureus* strain was sensitive to ampicillin and tetracycline before natural genetic transformation whereas it was resistance to these antibiotics after natural genetic transformation. *S. aureus* showed ampicillin and tetracycline resistance after transformation and showed plasmid DNA band which was absent in untransformed strain of *S. aureus* (Fig.5). Further work will be required to confirm the plasmid pBR322 DNA in transformed strain.

Numerous studies have shown that bacterial pathogens are increasingly gaining antibiotic resistance characteristics which can results in reduction in the potential use of antibiotics. To our knowledge, this report is the first to provide direct evidence that biofilm grown *S. aureus* can be efficiently induced to become genetically competent for transformation. It has been earlier reported that the biofilm environments provide optimal conditions for quorum sensing systems as found with the induction of genetic competence in *Acenitobacter* sp. strain BD4 13 Hendrickx et al. (2003). The transformed *S. aureus* showed reduced biofilm production which is considered to be a virulence marker. The natural genetic transformation can be used to genetically modify bacterial pathogens in order to reduce the pathogenicity and to determine unknown gene functions in *S. aureus* by constructing mutants. Thus applying the knowledge of quorum quenching on the bacterial biofilms and modifying the pathogenic strains in biofilms genetically by natural genetic transformation can lead us to alternative approaches against biofilm forming pathogenic micro-organisms in both medicine and industry.

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Figure Legends

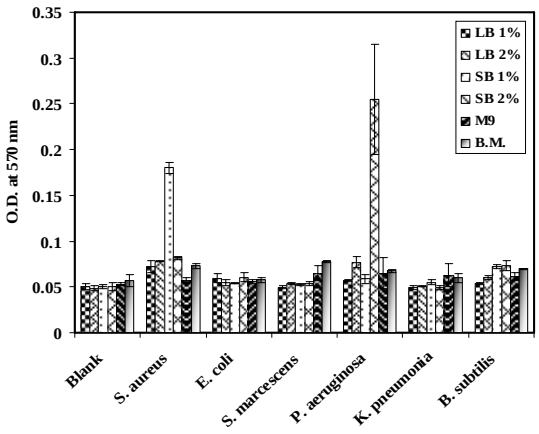


Fig. 1 Quantification (A 530) of biofilm formation of pathogenic strains in tryptone soy broth media in response to different concentration of glucose. Error bars represent the standard deviation of the mean A530 for nine independent wells.

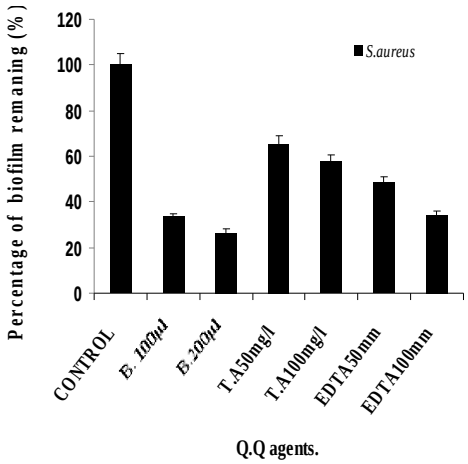
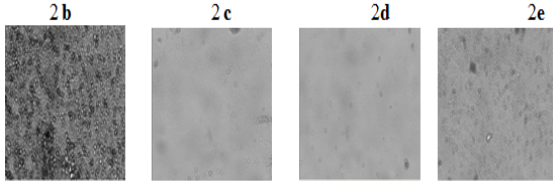


Fig. 2 a. Inhibition of biofilm formation of *S. aureus* by quorum quenchers at different concentrations. Cells were grown in tryptone soy broth media in the wells of polypropylene microtitre dishes. After incubation for 48 hrs at 37 ^{o}C planktonic cells were removed and attached cells were stained with crystal violet. Biofilm thickness in the absence of compounds is set to 100%. Error bars represent the standard deviation of the mean for six independent wells.



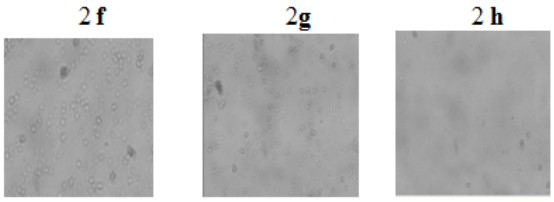


Fig. 2b, 2c, 2d, 2e, 2f, 2g and **2h**. Inverted microscopic images corresponding to the quorum quenching effects at 400X magnification.

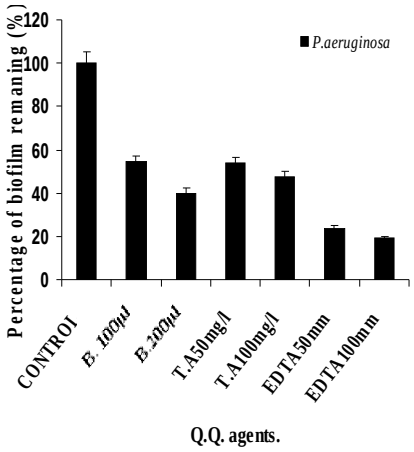


Fig. 3 a. Inhibition of biofilm formation of *P. aeruginosa* by quorum quenchers at different concentrations. Cells were grown in tryptone soy broth media in the wells of polypropylene microtitre dishes. After incubation for 48 hrs at 37 ^{o}C planktonic cells were removed and attached cells were stained with crystal violet. Biofilm thickness in the absence of compounds is set to 100%. Error bars represent the standard deviation of the mean for six independent wells.

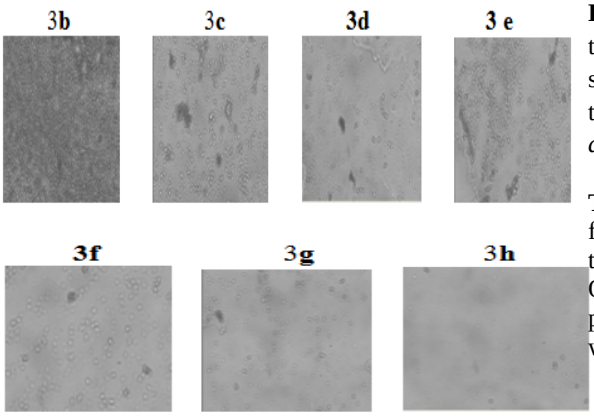


Fig. 3b, 3c, 3d, 3e, 3f, 3g and **3h**. Inverted microscopic images corresponding to the quorum quenching effects at 400X magnification.

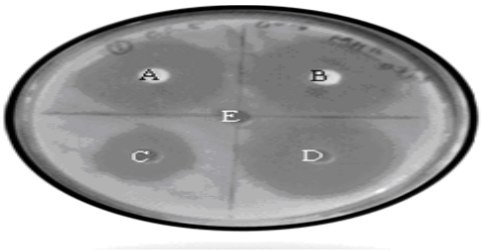


Fig. 4a

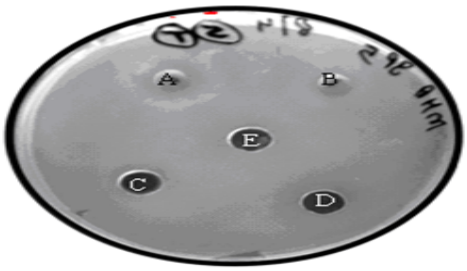


Fig. 4b

Fig. 4. Antibiotic susceptibility test for non-transformed **(a)** and transformed *S. aureus* **(b)**. A-ampicillin (2 µg), B - ampicillin (10 µg), C-tetracycline (10 µg), D-tetracycline (1000 µg) and E-blank (sterile water).

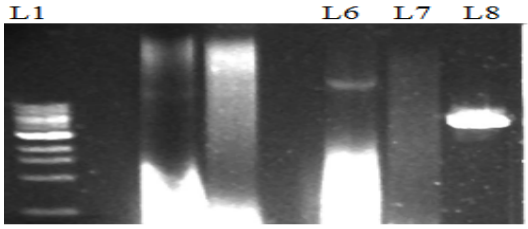


Fig. 5

Fig. 5 *S. aureus* showed plasmid DNA band after transformation which was absent in untransformed strain of *S. aureus* (Lane1-1 Kb ladder, Lane 6-transformed *S. aureus*, Lane 7- untransformed *S. aureus* and Lane 8- pBR322 DNA).

Table1: Screening of pathogenic strains for biofilm formation by tube, glass coupon reactor, congo red, tissue culture plate and plastic tube methods. Mean OD values < 0.120 was considered nonbiofilm producer, 0.120 – 0.240 was moderate and > 0.240 was considered as strong biofilm producers.

Table1:

Strains	Methods	Results
S. aureus MTCC 96	Tube method Glass coupon reactor method. Congo red method. Tissue culture plate method. Plastic tube method.	Positive Positive Positive O.D 570 nm -0.240 (moderate) Positive
P. aeruginosa MTCC 424	Tube method. Glass coupon reactor method. Congo red method. Tissue culture plate method. Plastic tube method.	Positive Moderate Negative O.D 570 nm -0.295 (strong) Positive
S. marcescens MTCC 86	Tube method. Glass coupon reactor method. Congo red method. Tissue culture plate method. Plastic tube method	Positive Positive Positive Positive Negative
E.coli NCIM 2065	Tube method. Glass coupon reactor method. Congo red method. Tissue culture plate method.	Negative Negative Negative Negative
B. subtilis NCIM 2045	Tube method. Glass coupon reactor method. Congo red method. Tissue culture plate method.	Negative Negative Negative Negative
K. pneumonia MTCC2696	Congo red method. Tissue culture plate method. Plastic tube method	Positive Negative Negative

Table2: Transformation efficiency obtained on transformation of *S. aureus* growing in one day **(2a)** and three day **(2b)** biofilms. The results are expressed as the mean value from three separate experiments $\pm$ standard deviation.

. **Table 2a**

One day biofilm	
Conc. of DNA used	**Transformation efficiency**
pBR_{322}plasmid DNA (0.5 μg/ml)	$8.6 \pm 0.7 \times 10^{3}$
pBR_{322} plasmid DNA (1.5 μg/ml)	$13.1 \pm 1.0 \times 10^{3}$

Table 2b

Three day biofilm	
Conc. of DNA used	**Transformation efficiency**
pBR_{322}plasmid DNA (0.5 μg/ml)	$1.6 \pm 0.6 \times 10^{3}$
pBR_{322} plasmid DNA (1.5 μg/ml)	$11.3 \pm 1.4 \times 10^{3}$