Pakistan J. Zool., vol. 51(6), pp 1-4, 2019. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2019.51.6.sc6

Short Communication

Biofilm Formation and Bacterial Aggregation Response of *Planomicrobium chinense* and *Alkaligenes faecalis* Associated with *Periplaneta americana* Found in Household Sewerage

Uzma Rafi*, Asmat, Muqaddes Niaz, Syeda Shazia Bokhari and Aisha Waheed Qurashi*

Department of Biology, Lahore Garrison University, Sector-C, Av-4, Phase VI, DHA Lahore

Article Information
Received 01 May 2017
Revised 11 July 2018
Accepted 13 February 2019
Available online 26 August 2019

Authors' Contributions
Asmat and MN performed the experiments as part of her thesis. UR and AWQ conceived the idea, supervised the research, wrote the manuscript. Shazia Bokhari helped in paper write up.

Key words
***Alkaligenes faecalis* (IM2), Auto aggregation, Biofilm, Cockroach, *Planomicrobium Chinense* (CXA1).**

ABSTRACT

Many bacterial species are harbored by cockroaches on the external and internal surfaces. These harbored species have great public health concern. There is a variable tendency of these microbes to adhere to various abiotic and biotic surfaces showing chances of infection. Present study aims to check biofilm formation and auto aggregation potential of the bacteria associated with cockroaches. For this purpose, cockroaches were captured from house sewerage and two bacterial strains CXA1 (*Planomicrobium chinense*) and IM2 (*Alkaligenes faecalis*) were isolated from external surface and rectum portion of cockroach, respectively. Bacterial autoaggregation started increasing after 1 h of culture incubation and reached maximum after 4 h. Isolate CXA1 showed maximum (136 %) auto aggregation as compared to isolate IM2 (37 %) after 4 h. However, biofilm formation in the isolate IM2 was higher as compared to isolate CXA1 at non shaking conditions. The study showed microbes with a potential to form biofilm are carried by insects and therefore there should be proper hygienic conditions and control efforts for the growth of these vectors.

Cockroaches are very common pest of dwelling places and carry different infectious microbes (Elyasigomari *et al*., 2017). Cockroaches generally prefer humid, warm, and dark areas like bathrooms, kitchens, and garages. Thus they are considered a very important vector of diseases including cholera and typhoid fever to humans (Ahmed *et al*., 2010; Shahraki *et al*., 2011). In the chronic diseases and wound infections formation of biofilms by pathogens appears to be an important factor (Omer *et al*., 2017). In most bacterial species for biofilm development and for autoaggregation many extracellular compounds which include flagella, exopolysaccharides (EPSs); surface component of bacteria and lipopolysaccharides (LPSs), in combination with environmental and quorum-sensing signals are essential (Hall-Stoodley and Stoodley, 2002; Kjelleberg and Molin, 2002). Bacterial aggregation of probiotics is a common mechanism for the successful survival and adhesion (Xu *et al*., 2009). Probiotic bacteria have been reported to show high auto aggregation that is helpful for surface colonization as reported previously by Janković *et al*. (2012) where auto aggregation ability of probiotic bacteria helps in bacterial adhesion to intestinal cells (Armas *et al*., 2017) and mammary glands (Espeche *et al*., 2009, 2012). The objective of the study was to evaluate the comparative efficacy of different microbial isolates associated with cockroaches towards aggregation and biofilm formation. Bacteria show variable response towards clumping and aggregation. Bacterial aggregation seems crucial steps for the development of biofilm formation however, many factors like synergism, antagonism or mutualism has been reported to influence it (Kolenbrander *et al*., 1985; Simoes *et al*., 2007). Bacterial aggregation can be auto aggregation or co-aggregation. If bacterial species of same strain adhere than this is auto-aggregation but if different species are involved in this interaction, this is co-aggregation. In earlier studies, many of the cell surface-associated structures like sugars have been found to play a role in bacterial co-aggregation resulting in biofilm formation (Rickard *et al*., 2003; Simoes *et al*., 2008). Research on insects present in dwelling places and associated microbes is significant because human health is at

* Corresponding author: aishawaheedqureshi@lgu.edu.pk; uzmazeeshan@lgu.edu.pk
0030-9923/2019/0006-0001 $ 9.00/0

great risk due to major challenge of microbial infection. The ability of cockroaches to explore different spaces in kitchen are associated with serious health concerns. Therefore control of insects is the need of the day as reported by many researchers. The present study is aimed to inspect the auto aggregation response and biofilm formation of bacteria associated with cockroaches. The bacterial auto aggregates formation although offers a benefit in term of bio augmentation (McLaughlin *et al*., 2006) however, in case of pathogenic bacteria, the chance for disease persistence increases. This study was designed with the aim to investigate auto aggregation in cockroach derived microbial communities so that the strategies can be developed to overcome these phenomena for preventing disease dispersal .

Material and methods

Cockroaches were trapped from house sewerage of Lahore, Pakistan in the month of March, 2016 and transported to laboratory for bacterial isolation. For the isolation of CXA1 from external surface, cockroaches were surface swabbed using sterile swab, while for isolation of IM2 isolate from internal surfaces, cockroaches were dissected using sterile dissecting tools and the digestive part was separated and serially diluted to isolate bacteria. Samples were finally spread on LB agar media (Gerhardt *et al*., 1994) and plates were incubated at 37°C for 24 h for bacterial growth. Isolated colonies were purified by quadrant streaking and screened for biofilm formation by mucoidy colony appearance and crystal violet ring formation assay (Christensen *et al*., 1985). Bacterial biofilm development in L-Broth culture was recorded after 48 h of culture incubation.

On the basis of their initial positive response towards biofilm formation bacterial isolates were characterized morphologically by following the methodology of Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) and biochemically (starch hydrolysis test, Citrate utilization test, Litmus milk reaction, Methyl Red Test, Voges Proskauer test, Catalase test) by following Tittsler and Sandholzer (1936). Molecular identification of bacterial isolates was done by analysis of 16S rRNA gene sequences done by commercially available ABI sequencing service Malaysia. Bacterial phylogenetic tree was constructed using neighbor joining method (Saitou and Nei, 1987) from the obtained nucleotide sequence with the help of MEGA 6.0 software (Tamura *et al*., 2013) to observe phylogenetic association. The obtained sequences were submitted in gene bank and the accession number was obtained.

Auto-aggregation assay was performed by following the method of Abdulla *et al*. (2014). Briefly, bacterial cells were harvested from the cultures (centrifugation at 2000 rpm for 20 min) grown in LB-Broth at 37°C for 24 h in shaking incubator. Harvested cell pellet was repeatedly washed and finally suspended in phosphate buffered saline. Auto aggregation response of bacterial isolates was determined by measuring absorbance at 600 nm from upper suspension after of every hour interval till five hours of incubation at room temperature.

For biofilm formation assay, quantification of bacterial biofilm formation of both isolates was determined in terms of tightly bound cells following the method of Liaqat *et al*. (2009) under shaking and non-shaking conditions after 144 h of culture incubation. Biofilm formation was determined in liquid media in test tubes and fresh bacterial cultures of CXA1 and IM2 were inoculated in L-Broth at equal cell densities $OD_{0.5}$ at 600 nm. Cultures were incubated at 37°C for 144 h and after incubation. The biofilm forming cells were stained using 0.01 % aqueous crystal violet solution after incubation. The stained cells were finally re dissolved in ethanol to get absorbance at 570 nm for biofilm formation. Bacterial biofilm was recorded as normalized values (OD 570/ OD 600) for excluding the difference in bacterial growth rate.

In all the experiments the data was analyzed statistically by calculating the mean values of replicates and measuring the standard errors of the means. The error bars are shown in each figure.

Results and discussion

The morphological characteristics of *Planomicrobium chinense* and *Alkaligenes faecalis* showed pigmented colonies, off white (*A. faecalis*) and orange (*P. chinense*), respectively. *A. faecalis* showed entirely flat colonies with Gram negative cocci cells. Cells showed no spores or capsules formation. *P. chinense* showed pin point colonies with smooth elevation (Supplementary Table I) while cells were gram positive, non-spore forming and non-capsulated rods (Supplementary Table II). *A. faecalis* showed positive result of catalase, starch, litmus milk reaction, methyl red while negative for Voges-Proskauer test. *P. chinense* showed positive results for catalase, litmus milk reaction while negative results were recorded for starch test, methyl red test and Voges-Proskauer test. On the basis of 16 S rRNA gene sequence homology with data base of Genebank at National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov), carried out by Basic Local Alignment Search Tool (BLAST), the phylogenetic tree construction showed that there were two clusters in a phylogenetic tree and both isolates showed strong homology with their compared nucleotide sequences (Supplementary Fig. 1). Morphological characteristics like shape, flagellar arrangement and staining are the important parameters for bacterial identification (Tshikhudo *et al*.,

2013). Cockroaches carry many pathogenic and non-pathogenic bacteria. Both the isolates were mucoidy in appearance and this is in line with previous experiments showing that exposure to stress like antibiotic results in induction of mucoidy phenotypes resulting in a thick biofilm development (Supplementary Fig. 2) (Kaplan, 2011; Weiser *et al*., 2016; Mlynek *et al*., 2016). Moreover, exopolymeric production in mucoidy phenotype might have resulted in enhanced biofilm formation as indicated by previous *in vivo* and *in vitro* experiments showing that the exopolymer protects chronic wound biofilms (Thurlow *et al*., 2011).

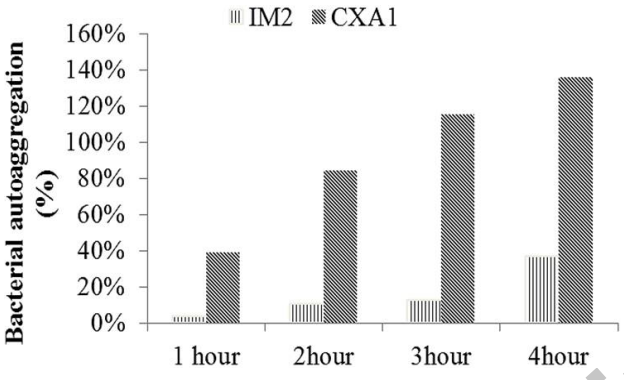

Fig. 1. Bacterial auto aggregation response at different time intervals.

The percentages of auto aggregation increased with the passage of time and increased to 37 % after four hours of culture incubation in *A. faecalis* while in *P. chinense* auto aggregation increased to 136% after four hours of culture incubation (Fig. 1). Increased in autoaggregation with the passage of time shows that aggregation is a time dependant phenomenon these results are supported by the work of Ramalingam *et al*. (2013) where visible aggregates appeared after 72 h of incubation. Bacterial aggregation is a reversible process observed in bacterial cells and it is directly correlated with the bacterial adhesion and colonization. Bacterial coaggregation and auto aggregation is a common feature reported in many bacterial species (Jankovic *et al*., 2012). Auto aggregations in probiotic bacteria offer them additional benefits of successful survival by excluding the species commonly present for pathogenesis. This has been reported to be an important criterion for the selection and screening of probiotic bacteria. However, the variable response of auto aggregation in both isolates shows their efficacy to auto aggregate either due to being different species or their isolation source. This is in line with previous findings where *Flavobacterium johnsoniae* displayed variation in autoaggregation with Gram-negative and Gram-positive organisms (Basson *et al*., 2008).

In general the biofilm formation was highest in *A. faecalis* compared to *P. chinense* (Fig. 2). The biofiolm formation was highest in non-shaking condition in *A. faecalis;* however, biofilm formation was maximum in *P. chinense* as compared to other strain (Fig. 2). Biofilm formation was checked by both isolates and it was noted that that both isolates have ability to form biofilm. The normalized values of biofilm are supportive for describing relation of biofilm to bacterial cell mass. This has been especially described under growth limiting conditions for bacterial cells. The results of present study showed that presence of cockroaches at dwelling places should be avoided because the bacteria have high colonization ability. Future implication of this work is quite promising for reducing diseases associated with cockroaches. An additional benefit of autoaggregation to microbes associated with cockroaches is that they escape the process of grazing and avoid the loss in cell numbers (Cray *et al*., 2013). These microbes in the colon portion of cockroach and external cuticle have prevented themselves from external stress factors like enzymes or disinfectants by increasing the rate of auto aggregation.

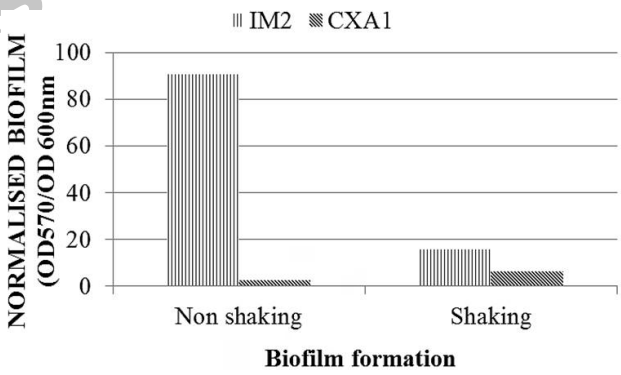

Fig. 2. Bacterial biofilm formation at shaking and non-shaking conditions.

Since the aggregated form of the cockroach derived microbes have shown potential to form biofilm, detailed molecular analysis of this work will be helpful for disease eradication and control.

Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2019.51.6.sc6

Statement of conflict of interest

The authors declare no conflict of interest.

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Supplementary Material

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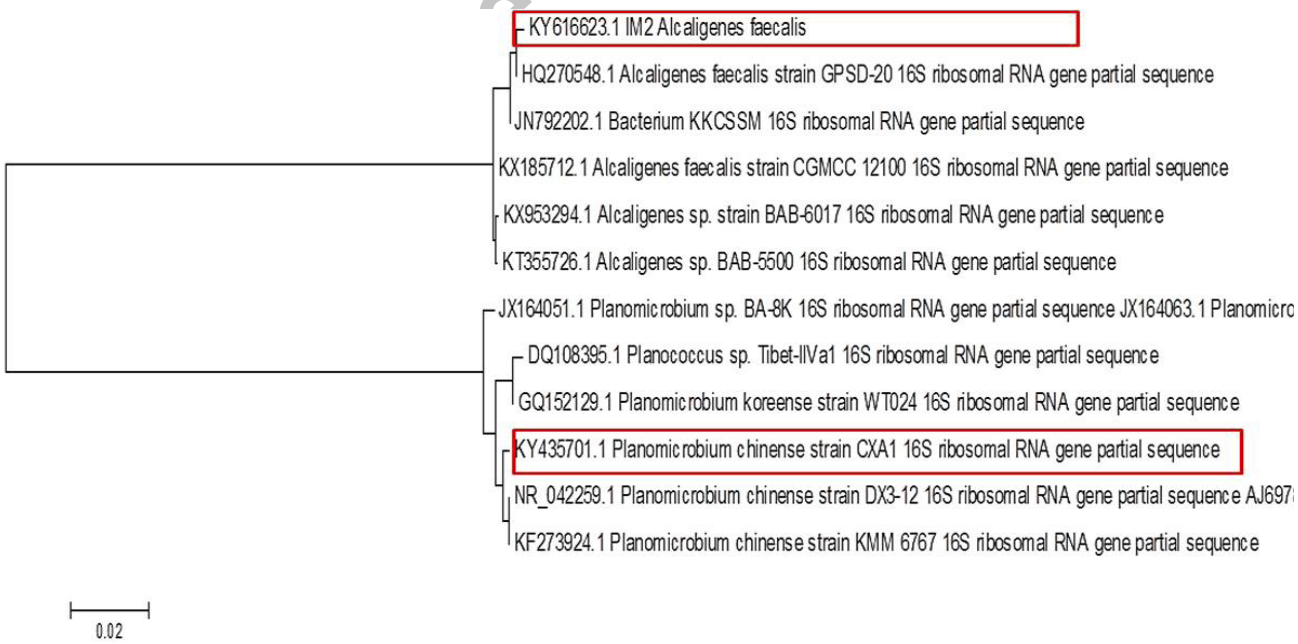
Uzma Rafi*, Asmat, Muqaddes Niaz, Syeda Shazia Bokhari and Aisha Waheed Qurashi*

Department of Biology, Lahore Garrison University, Sector-C, Av-4, Phase VI, DHA Lahore

* Corresponding author: aishawaheedqureshi@lgu.edu.pk; uzmazeeshan@lgu.edu.pk
0030-9923/2019/0006-0001 $ 9.00/0

Supplementary Table I. Morphological and cultural characterization of isolates.

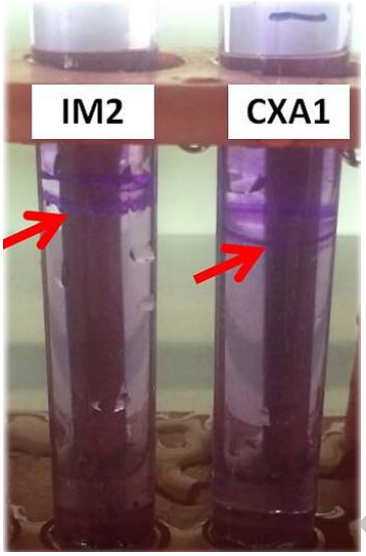
Strains	Culture characteristics					Staining results			
	Appearance	Form	Pigmentation	Margin	Elevation	Simple Staining	Gram staining	Spore staining	Capsule staining
IM2 (*Alkaligenes faecalis*)	Mucoidy	Circular	Off White	Entire	Flat	Coccus	Gram Positive	Negative	Negative
CXA1 *(Planomicrobium chinense)*	Mucoidy	Circular	Orange	Pin point	Flat	Rods	Gram Positive	Negative	Negative



Supplementary Fig. 1. Phylogenetic analysis based on 16 S rRNA showing homology to other reference nucleotide sequences was derived using MEGA6 software. The analysis is based on neighbor join method involving 12 nucleotide sequences. For each of the strains Gene Bank accession numbers are provided.

Supplementary Table II. Biochemical characterization of isolates.

Strains	Biofilm ring assay	Starch Hydrolysis	Citrate utilization test	Litmus milk reaction	Methyl red test	Voges-Proskauer test	Catalase test
IM2 (*Alkaligenes faecalis*)	Positive	Positive	Positive	Acid with Reduction	Positive	Negative	Positive
CXA1 *(Planomicrobium chinense)*	Positive	Negative	Positive	Acid with Reduction	Negative	Negative	Positive



Supplementary Fig. 2. Biofilm Ring Assay: Crystal violet ring was formed after 144 h of culture incubation.