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Effects of plasmid acquisition by *Pseudomonas aeruginosa* clinical isolates on methicillin-resistant *Staphylococcus aureus* biofilm formation

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This work aimed to study the influence of plasmid acquisition on the mutual interaction of certain *Pseudomonas aeruginosa* clinical isolates and methicillin resistant *Staphylococcus aureus* (MRSA) clinical isolates. In this study, two mixed cultures were used: i) *P. aeruginosa* clinical isolate P14 which co-existed with MRSA isolate S14; and ii) *P. aeruginosa* clinical isolate P12 which co-existed with MRSAisolate S12. The respective mixed cultures were selected to study the existence of the plasmids on the inhibitory effect of the culture supernatant of *P. aeruginosa* on the biofilm formation of co-existing MRSA clinical isolates. The culture supernatant of *P. aeruginosa* harboring no plasmids exerted a significant reduction effect on the biofilm formation (about 57% reduction; P < 0.005) of the co-existing MRSA isolate and such effect was also confirmed using plasmid transformation experiments. To ensure that such effect was habitually due to the presence of plasmids and not to any mutational or conformational changes in the gene/protein sequences of the key genetic elements involved in interspecies interaction, the genes, *araC*, *pqsS* and *ahlS*coded for AraC family transcriptional regulator (1026 bp, 341 aa), and 2-heptyl-3-hydroxy-4(1H)-quinolone synthase (1149 bp, 382 aa) and acyl-homoserine-lactone synthase (540 bp; 179 aa) of *P. aeruginosa* clinical isolate P14 were amplified using PCR, analyzed and submitted into the GenBank database under the accession codes, KT693035, KT693034, KT693033, respectively. AraC was a model of AraC transcription regulator with a conserved N-terminal arabinose-binding domain and C-terminal H-T-H motive. PqsS was a model of putative 2-heptyl-3-hydroxy-4(1H)-quinolone synthase with a conserved domain of a NAD(P)-binding Rossmann-like domain. AhlS showed a conserved domain with the acyl-homoserine-lactone synthase (LasI) of the protein family COG3916. The open reading frames (ORFs) of the respective genes showed no mutation or deviation in the predicted tertiary structures.Inconclusion, plasmid acquisition significantly decreased the inhibitoryeffect of *P. aeruginosa* on the biofilm formation of the co-existing MRSA isolates.

Key words: Arabinose-binding transcription regulator, 2-heptyl-3-hydroxy-4(1H)-quinolone synthase, acyl-homoserine-lactone synthase, *Pseudomonas aeruginosa,* methicillin resistant *Staphylococcus aureus* (MRSA), biofilm, Quorum sensing.

INTRODUCTION

Microbial infections of many anatomical sites have been investigated to be polymicrobial where several molecules and signal peptides are involved in the interspecies interaction of the co-infected pathogens (Hoffman et al.,

2006; Schertzer et al., 2009; Schuster et al., 2013; Fugère et al., 2014; Rüger et al., 2014; Castillo-Juárezet al., 2015).Mixedinfections were found to be more common among hospitalized and immunosuppressed patients where the opportunistic pathogens *P. aeruginosa* and *S. aureus* commonly co-excisted (Brogden et al., 2005; Hoffman et al., 2006). Both organisms are commonly co-isolated from cystic fibrosis respiratory cultures where *P. aeruginosa* competes with *S. aureus* and produces an antistaphylococcal substance, 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO), which suppresses the growth of many Gram-positive bacteria (Machan et al., 1992; Hoffman et al., 2006). Over the last 10 years, significant progress has been made in elucidating the molecular mechanisms underlying *P. aeruginosa* pathogenicity including the chemical and biological activities of certain molecules and regulatory proteins involved in quorum sensing as well as the formation of biofilms (Kaufmann et al., 2005; Fugère et al., 2014).

Methicillin resistant *S. Aureus* (MRSA) is one of the most common bacterial pathogens responsible for the hospital acquired infections in Egypt and its management has become increasingly a major problem. Therefore, interspecies interaction of microbes that coexisted is an important step towards exploring new molecules that can become potential therapeutics, particularly those targeting bacterial virulence such as biofilm formation (Jevon et al., 1999; Donlan and Costerton, 2002). The strategy of many antivirulence drugs is to target specific virulence determinants that play a key role in the microbial pathogenesis, thus inhibiting specific mechanisms that promote pathogenesis and infection (Marra, 2000; Rubinsztein et al., 2005; Torres et al., 2005). Many studies have recently been conducted to explore microbe-microbe interactions among those coexisting in various infectious sites; however results showed that this type of interaction was a multifactorial process and was variable among different microbial species (Kadurugamuwa and Beveridge, 1996; Hoffman et al., 2006; Manefield and Whiteley, 2007; Atkinson and Williams; 2009; Riedele and Reicht, 2011; Fugère et al., 2014; Rüger et al., 2014). Therefore, the present study aimed to study the influence of plasmid acquisition ofcertain*P.aeruginosa* clinical isolates on the biofilm formation of methicillin resistant *S. aureus* (MRSA) that wereco-isolated from pus specimens. This effect was confirmed using plasmid transformation experiments as well as analysis of certain genes whose gene products were previously confirmed to be involved in the regulation and synthesis of some relevant quorum sensing signal molecules of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and culture media

Two mixed cultures: *P. aeruginosa* clinical isolate P12 coexisting with methicillin resistant *S. aureus* (MRSA) isolate S12; and *P. aeruginosa* clinical isolate P14 coexisting with MRSA isolate S12 were recovered from pus specimens collected from infected wound of two patients. The collected isolates were cultured on tryptic soy broth (TSB) or on solid or liquid LB culture medium at 37°C (Kieser et al., 2000).

Bio film assay by spectrophotometric method

This was carried out according to Christensen et al. (1985) and Wakimoto et al. (2004). To evaluate the effect of *P. aeruginosa* supernatants on biofilm formation by *S. aureus*, the culture supernatants were filter sterilized and about 150 µl were distributed in each well of a 96-well tissue culture flat-bottom polystyrene microtiter plate (BD). Then, aliquots (150 µl) of *S. aureus* suspension were added to each well of the plate already containing the *P. aeruginosa* supernatant dilutions. The plates were incubated for 48 h at 37°C. The cultures were then discarded and the wells were carefully washed three times with 200 µl PBS/well. Once dried, the plates were treated for 30 min with 200 µl (in each well) of a 2% crystal violet solution to stain the biofilm, rinsed twice with 200 µl water to remove excess dye, and then allowed to dry. The absorbance of each well at 590 nm was measured. The assays were repeated three times with eight replicates for each condition tested (Fugère et al., 2014).

Chromosomal DNA extraction from the clinical isolates

Chromosomal DNA of certain clinical isolate was extracted using Gene Jet Genomic DNA purification kit (Thermo Scientific, UK) according to the manufacturer‘s specifications.

Plasmid extraction from certain clinical isolates

Plasmids were extracted by GeneJet Plasmid Miniprep Kit according to the manufacturer‘s specifications. The recovered plasmids were detected using agarose gel electrophoresis (Sambrook and Russell, 2001).

Agarose gel electrophoresis

Chromosomal DNA and/or extracted plasmids were detected using agarose gel electrophoresis (Sambrook and Russell, 2001). The DNA fragment size was determined according to its migration distance within the gel in comparison with a parallel migrated DNA ladder.

Preparations, transformation and regeneration of competent *P. aeruginosa* clinical isolate P14 cells

Preparations of competent *P. aeruginosa* clinical isolate P14 cells were performed using the standard protocols of Hanahan (1983). *P.*

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Table 1. Primers used in this study.

S/N	Primers	Annealing temperature	Expected PCR product size (kb)	Reference
1	PfahlF- 5'ATGCAAACTTTCGTTCACGAGGCAGGC-3' PRahlF 5'GTCGATCCAGCATGCCACCACC-3'	63	0.54	This work
2	pqsH-F:5'-ATGACCGTTCTTATCCAGGGG-3' pqsH-R: 5'-CTACTGCGCGGCCATCTCAC-3'	59	1.2	This work
3	Pfarc F 5'-atgatccctgcaacccgcattac-3' PRarc-R 5'CAATTGGCATGCTGACGCCGAT-3'	60	0.66	This work
4	PRarc-R 5'CAATTGGCATGCTGACGCCGAT-3' pqsH-R: 5'-CTACTGCGCGGCCATCTCAC-3'	57	2.1	This work

aeruginosa clinical isolate P14 cells were transformed according to and Russell (2001). In general, 10 µl oftheextracted plasmids from *P. aeruginosa* clinical isolate P12 cellscontaining 0.1 - 1.0 µg DNA were added to 200 µl competent cells and kept on ice for 30 min. Following a heat shock (90 s, 42°C), cells were regenerated in 800 µl SOC or LB medium for 4 h at 37°C, 300 rpm then the cell suspension was plated out on LB agar plates containing the appropriate antibiotic. Transformants were selected and plasmid extractions were carried out and analyzed using AGE.

Amplificationofsome resistance genes by PCR

Primers design

The genes reported in the literature responsible for 2-heptyl-3-hydroxy-4(1H)-quinolone synthase, AraC family transcriptional regulator and Acyl-homoserine-lactone synthase of *P. aeruginosa* were assigned. The National Center for Biotechnology Information database (NCBI) (http://www.ncbi.nlm.nih.gov/)wassearched for the desired genes and their translated proteins. The multiple alignments, for the nucleotides and the amino acids of selected genes against other alleles in NCBI database, were carried out using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).Primerswere designed using the conserved regions of the amino acid alignment data at the beginning and the end of the gene/protein sequences. The analysis of the primers was performed using pDRAW32 program (http://www.acasoft.dk/acaclone/download/install.htm) and the melting temperatures (T_m) were calculated. The check for the absence of self-complementarily or formation of heterodimers by the designed primers and the confirmation of the target sequences were carried out using Primer-BLAST on the NCBI database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).Primersused in this study are listed in Table 1.

Polymerase chain reaction (PCR)

Amplification of the selected genes was carried out via PCR using the designed primers. Chromosomal DNA and/or plasmids extracted using Gene Jet plasmid miniprep kit were used as templates for PCR which was performed in a NYX TECHNIK ATC 40I Thermocycler, USA. Amplification of different genes by PCR was performed using 200 – 400 ng of the genomic DNA as a template and the selected primers for each probe (Table 1). PCR was performed in a Nyx-Technik Inc. Personal Cycler (ATC401, USA). Each assay (50 µl) consisted of 200 ng chromosomal DNA, 50 pmole of each appropriate primer, 25 µl of DreamTaq™ Green PCR master mix (2X). PCR general conditions were: 95°C for 4 min; then 30 cycles [95°C for 30 s; annealing temperatures and time according to Table 1 for 45 s, 72°C for 45 s (normally 1 min for 1 kb)]; and 72°C for 10 min (ramping rate 1°C/s).

The PCR products obtained on agarose gel were excised and subsequently purified using AxyPrep DNA Gel Extraction Kit (Axygen Scientific, Inc., USA) according to the manufacture's specifications.

DNA sequencing, assemble and detection of possible open reading frames (ORFs)

The PCR products were purified using GeneJET^{TM}purification kit at Sigma Scientific Services Company, Egypt. Then, they were sent for sequencing at GATC, Germany using ABI 3730xl DNA sequencer. The PCR products were sequenced from both forward and reverse directions. The obtained sequence files were assembled into a final contig using StadenPackage program version 3 (http://staden.sourceforge.net/) (Staden,1996). The resulted contig was analyzed for detection of ORFs using FramePlot 2.3.2 (http://www0.nih.go.jp/~jun/cgi-bin/frameplot.pl) (Ishikawa and Hotta, 1999), annotated and submitted into the GenBank database. Restriction analysis of the final contig was carried out using Restriction Mapper version 3 (http://www.restrictionmapper.org).

Nucleotide accession codes

Nucleotide sequences of *ahl*S, *pqs*S and *ara*C obtained in this study were submitted to the GenBank database under the accession codesKT693033, KT693034 and KT693035, respectively.

Protein analysis and detection of the conserved domains of encoded proteins

Multiple alignment and phylogeny analysis of amino acids of the obtained ORFs were carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2 (Thompson et al., 1994). Structure of proteins and conserved domain analysis were conducted using Basic Local Alignment Search Tool (NCBI):

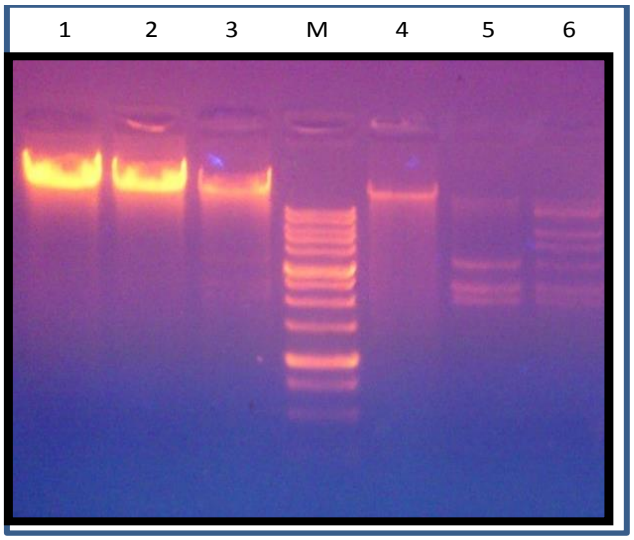

Figure 1. Agarose gel electrophoresis of: A. Chromosomal DNA extracted from *P. aeruginosa* clinical isolate P14 (lane 1); *P. aeruginosa* clinical isolate P14 after being transformed with plasmids extracted from *P. aeruginosa* clinical isolate P12 (P14t; lane 2) and *P. aeruginosa* clinical isolate P12 (lane 3); B. Plasmid extract of *P. aeruginosa* clinical isolate P14 (lane 4); *P. aeruginosa* clinical isolate P14 after being transformed with plasmids extracted from *P. aeruginosa* clinical isolate P12 (lane 5) and *P. aeruginosa* clinical isolate P12 (lane 6).Lane "M" is 1.0 kb DNA ladder (Thermo Scientific, USA).

http://www.ncbi.nlm.nih.gov/Structure/index.shtml (Marchler-Bauer et al., 2015).

Analysis and prediction of the tertiary structure of encoded proteins

The putative tertiary structure of the encoded proteins were analyzed and predicted using Swiss-Model software (http://swissmodel.expasy.org(Arnoldet al., 2006, Kiefer et al., 2009; Guex et al., 2009; Biasini et al., 2014). The QMEAN4 score of predicted protein model was also calculated (Benkert et al., 2011). This was done to show the predicted conformation of the protein and the possible metal/legand-binding residues which might have an effect on the protein activity.

RESULTS

Chromosomal and plasmid DNA extraction from the clinical isolates

Chromosomal DNA and plasmid DNA extracted from different *P. aeruginosa* clinical isolates areshown in Figure 1.

Biofilmassay

Results of biofilm assay of *S. aureus* clinical isolate S14 or S12 alone; *P. aeruginosa* clinical isolate P14, P14 transformant (P14t) or P12 alone; *S. aureus* clinical isolate S14 or S12 in the presence of culture supernatant of *P. aeruginosa* clinical isolate P14 or P12 or P14t; and *P.aeruginosa*clinical isolate P14 or P12 in the presence of culture supernatant from *S. aureus* clinical isolate S14 or S12 are depicted in Figure 2.

The genetic analysis of the genes/enzymes that were previously documented to be involved in the regulation and synthesis of major molecules of quorum sensing of *P. aeruginosa*clinical isolate P14particularly when coexisting with Gram positive pathogens have been studied. This was carried outto ensure absence of any mutational or conformational changes of the nucleotide or amino acid sequences of the key genes/gene products involved in microbe-microbe interaction. These genes/enzymes include, AraC family transcriptional regulator, 2-heptyl-3-hydroxy-4(1H)-quinolone synthase (PqsS) and acyl-homoserine-lactone synthase (AhlS).

Sequenceanalysis of the DNA segment (final contig) harboring both *ara*C and *pqs*S

As depicted in Figure 3, two open reading frames (ORFs) were detected and annotatedAraC and PqsS on the submitted DNA segment (2.208 kb) of *P. aeruginosa* clinical isolate P14. The *araC* (1026 bp) and *pqsS* (1149 bp) genes were found to encode a predicted AraC family transcriptional regulator of 341 amino acids (aa) and a predicted 2-heptyl-3-hydroxy-4(1H)-quinolone synthase of 382 aa, respectively. PqsS was encoded by the parent DNA strand while AraC was encoded by the complementary strand and located upstream PqsS. A possible strong ribosomal binding site (RBS) for each ORF was detected and annotated as 5'-GAAGAGG-3' located at position -7 from the predicted start codon (ATG) of AraC and 5'-GGAGAG-3' located at position -5 from the predicted start codon (ATG) of PqsS. Restriction analysis profile of the respective DNA segment using some selected restriction endonucleases was also illustrated (Figure 3).

Multiple alignments and domain analysis of AraC (predicted AraC family transcriptional regulator) with homologous proteins

As shown in Figure 4, AraC showed more than 88, 86, 90 and 84% similarities in the amino acid sequences of homologous proteins encoding diverse AraC transcription regulators of AraC family (accession code, AC= Q5LUJ7), *Stackebrandtia nassauensis* DSM 44728 (AC= YP_003512645), *Burkholderia ambifaria* MC40-6 (AC = YP_001815544) and *Desulfatibacillum alkenivorans* AK-01 (AC = YP_002433305), respectively. The AraC and the respective homologous proteins were highly conserved more than 92% at the N-terminal (arabinose

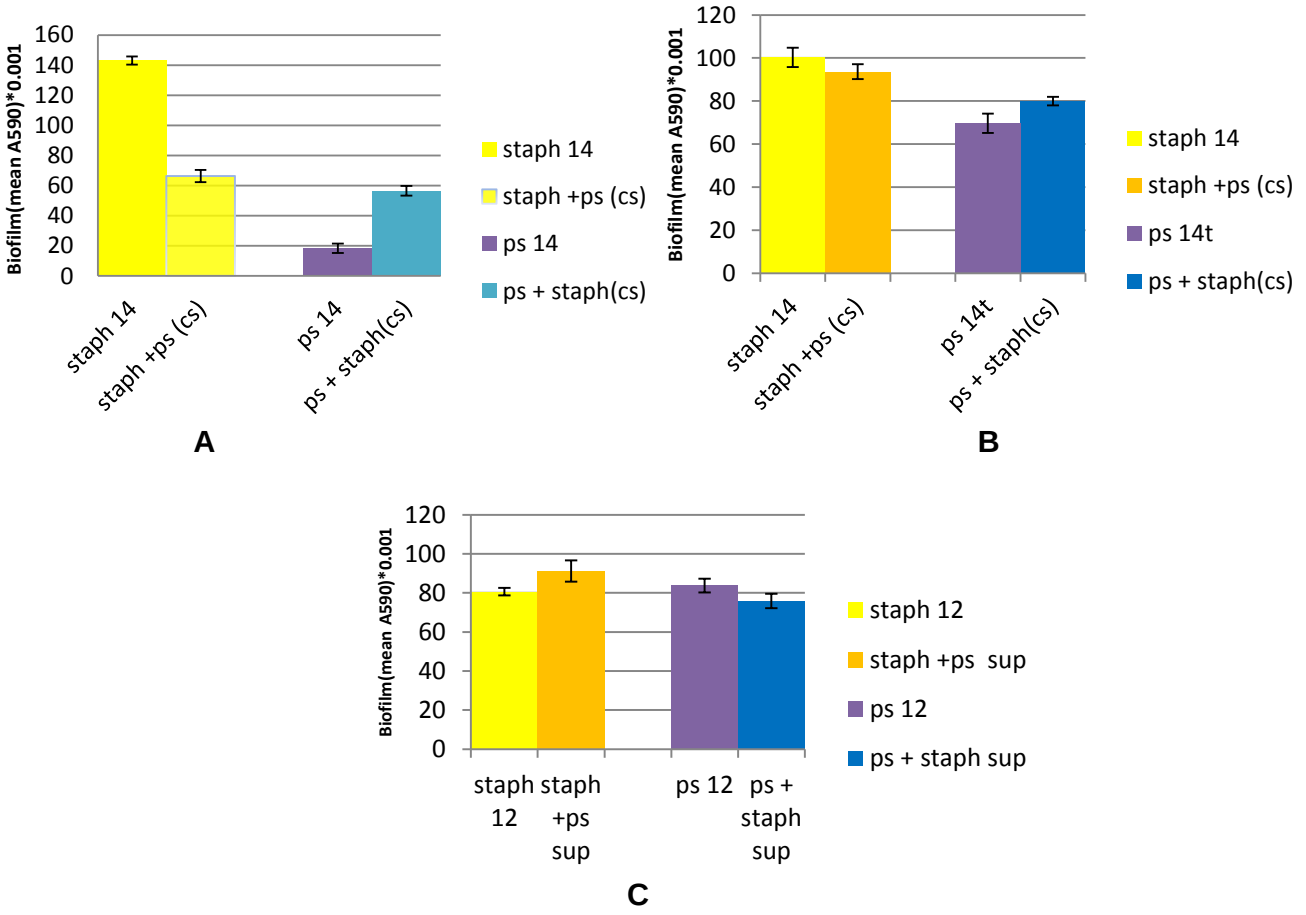

Figure 2. Results of biofilm assay using: A, mixed culture of *P. aeruginosa* isolate P14 and *S. aureus* isolate P14; B, mixed culture of *P. aeruginosa* isolate P14 transformant and *S. aureus* isolate P14; C, mixed culture of *P. aeruginosa* isolate P12 and *S. aureus* isolate P12. Each organism alone or with the culture supernatant (cs) of the other co-existingorganism.

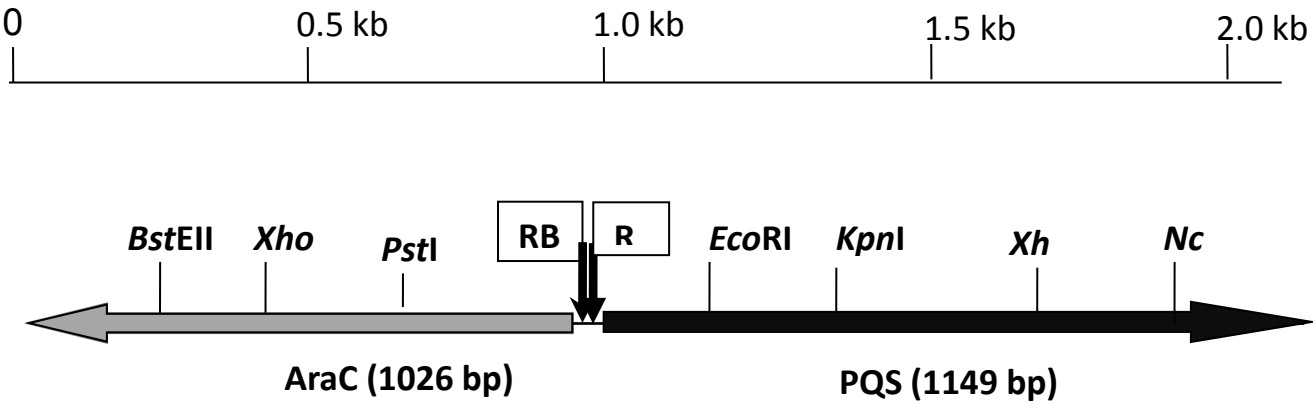

Figure 3. Restriction analysis profile of the DNA segment of *P. aeruginosa* isolate P14 submitted to GenBank database. AraC= predicted AraC family transcriptional regulator (1026 bp, 341 aa; AC=KT693035), PqsS = 2-heptyl-3-hydroxy-4(1H)-quinolone synthase (1149 bp, 382 aa; AC=KT693034). Arrows indicate direction of the open reading frames (ORFs). Abbreviations: AC = accession code; RBS = possible ribosomal binding site.

binding domain) and at the C-terminal residues (Helix Turn-Helix, H-T-H motive required for DNA binding).

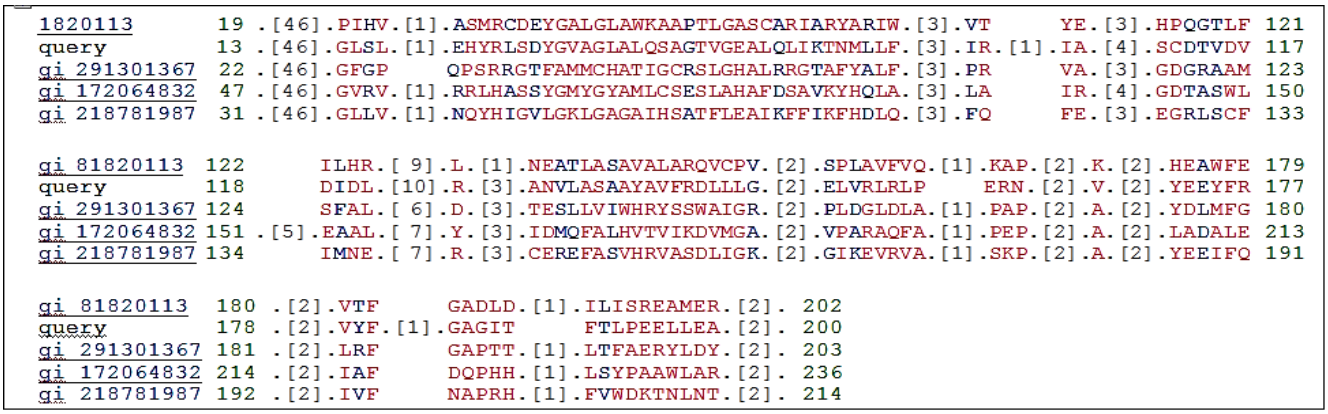

Figure 4. Multiple amino acid sequences of the conserved domains of the AraC transcriptional regulator protein of *P. aeruginosa* isolate P14 (AraC query) and its homologous using http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi (Marchler-Bauer et al., 2015). The numbers indicate the position within the corresponding proteins: gi 81820113 = transcriptional regulator AraC family of *Ruegeriapomeroyi*, accession code (AC)= Q5LUJ7; gi 291301367 = AraC family transcriptional regulator of *Stackebrandtianassauensis* DSM 44728, AC= YP_003512645; gi 172064832 = AraC family transcriptional regulator of *Bu. ambifaria* MC40-6, AC= YP_001815544; gi 218781987 = AraC family transcriptional regulator of *Desulfatibacillumalkenivorans* AK-01, AC = YP_002433305.. Amino acids aligned to form position specific scoring matrix (PSSM) where red or blue colors representing degree of conservation with red designating highly conserved. Dashes indicate variation in sequence length among aligned proteins.

Multiple alignment and domain analysis of predicted 2-heptyl-3-hydroxy-4(1H)-quinolone synthase (PqsS)with homologous proteins

As shown in Figure 5, PqsS showed more 83% similarities in the amino acid sequences with itshomologous proteins encoding diverse 2-heptyl-3-hydroxy-4(1H)-quinolonesynthase proteins of *Acinetobacter* sp. ADP1 (AC= YP_046465), *Meiothermus ruber* DSM 1279 (AC= YP_003508322), *Rubrobacter xylanophilus* DSM 9941 (AC= YP_644990), *Bacillus subtilis* subsp. *subtilis* str. 168, Rossmann-fold NAD(P)(+)-binding proteins; cl21454, (AC= NP_388604).Domain analysis of PqsS protein revealed a putative conserved domain of the Rossmann-fold NAD(P)(+)-binding protein (AC=cl21454).

DNA segment(finalcontig)harboringacyl-homoserine lactone synthase (AhlS), multiple alignment and domain analysis

Asdelineatedin Figure 6, open reading frames (ORFs; partial 3') was detected and annotated AhlS on the submitted DNA segment (540 bp) of *P. aeruginosa* clinical isolate P14. The AhlS was found to encode a predicted acyl-homoserine lactone synthase. AhlS showed more 85% similarities in the amino acid sequences with itshomologous proteins encoding diverse acyl-homoserinelactone synthase proteins of a symbiotic bacterium *Mesorhizobium loti*, accession code (AC)= NP_106905; *P. aeruginosa* (LasI; AC= LASI_PSEAE; Gould et al., 2004), *P. aeruginosa* (RhlI; AC= RHLI_PSEAE) and *Ralstonia solanacearum* (SolI; AC= SOLI_RALSO) (Figure 7). AhlS protein revealed a putative conserved domain of N-acyl-L-homoserine lactone synthetase (accession: COG3916) as well as with the autoinducersynthetase of the protein family, pfam00765.

Phylogram analysis of AraC, PqsS and AhlS

Cladogram analysis outlined in Figure 8 showing AraC in relation to other diverse transcription regulator proteins. AraC of *P. aeruginosa* isolate P14 clustered closely with a homologous protein of AraC family, accession code(AC= Q5LUJ7)ofpairwise score ranged from 0.40122-0.40127 and was relatively related to other distinct clusters (pairwise scores ranged from 0.34135-0.40127). PqsS of *P. aeruginosa* isolate P14 was clustered closely with an oxidoreductase of *B. subtilis* subsp. *subtilis*str. 168, with Rossmann-fold NAD(P)(+)-binding protein (AC= NP_388604)of pairwise score ranging from 0.35279-0.39583 (Figure 9).AhlS of *P. aeruginosa* isolate P14 was clustered closely with Acyl-homoserine-lactone synthase, an autoinducer synthesis protein of *P.aeruginosa* (AC= RHLI_PSEAE) of pairwise score ranged from 0.00145-0.01821 (Figure 10).

Prediction of the tertiary structure of the AraC, PqsS and AhlS proteins using SWISS-MODEL homology modeling report

Asdepicted in Figure 11, the tertiary structure of AraC

Figure 5. Multiple amino acid sequences of the putative 2-heptyl-3-hydroxy-4(1H)-quinolone synthase of *P. aeruginosa* isolate P14(PqsS, query) and its homologous using http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi (Marchler-Bauer et al., 2015). The numbers in green indicate the position within the corresponding proteins: gi 50084955 = 2-heptyl-3-hydroxy-4(1H)-quinolone synthaseprotein of *Acinetobacter* sp. ADP1, accession code (AC)= YP_046465; gi 291296924 = monooxygenase FAD-binding protein of *Meiothermusruber* DSM 1279, AC= YP_003508322; gi 108805053 = FAD-binding FAD dependent oxidoreductase of *Rubrobacterxylanophilus* DSM 9941; AC= YP_644990; gi 16077790 = oxidoreductase of *Bacillus subtilis* subsp. *subtilis*str. 168, Rossmann-fold NAD(P)(+)-binding proteins; cl21454, AC= NP_388604. Amino acids aligned to form position specific scoring matrix (PSSM) where red or blue colors represent degree of conservation with red designating highly conserved. Dashes indicate variation in sequence length among aligned proteins.

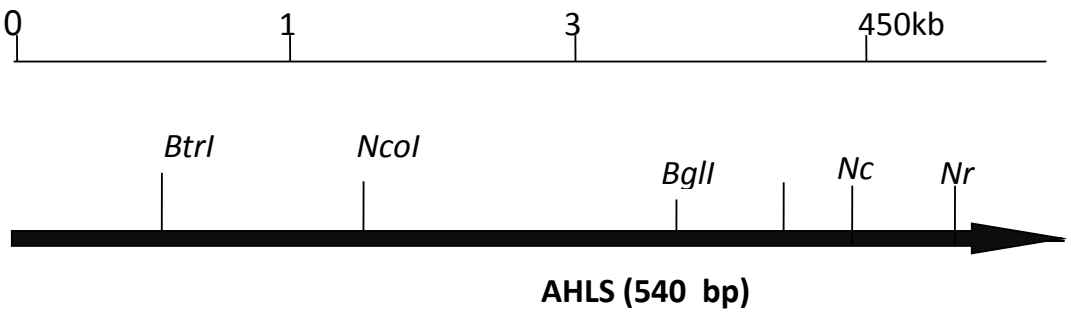

Figure 6. Restriction analysis profile of the DNA segment coded for Acylhomoserine lactone of *P. aeruginosae* isolate P14 (Partial AhlS; 540 bp; 179 aa) submitted to GenBank database (accession code: KT693033) using Restriction Mapper version 3 (http://www.restrictionmapper.org). Arrows indicate direction of the open reading frames (ORF).

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gi 13475341  12 .[2].QLITPGLYSEFAGELK     EMHGLRYRVFKERLDWEVQTGG     E     METDTFDDLKPVYLLL.[1]. 69
query         1 .[2].ELLSESLEGLSAAMIA     ELGRYRHQVFIEKLGWDVVSTS.[3].D     QEFDQFDHPQTRYIVA.[1]. 61
gi 462480     1      MIVQIGRREEFDKKLL.[1].EMHKLRAQVFKERKGWDVSVID     E     MEIDGYDALSPYYMLI.[3]. 59
gi 12230962   1 .[2].ELLSESLEGLSAAMIA     ELGRYRHQVFIEKLGWDVVSTS     R.[3].QEFDQFDHPQTRYIVA      60
gi 20140007   1      MQTFIHGGGRLPEAVD.[1].ALAHYRHQIFVGQLGWQLPMAD     G.[1].FERDQYDRDDTVYVVA.[1]. 58

gi 13475341  70 GSDW.[1].IRGCVRLLPTTGPTMLRDTFPALLG.[2].VAPASPDIWESSRF.[9].KAAGGLAQ.[2].YELFAGMIE 143
query        62 GRQG     ICGCARLLPTTDAYLLKEVFAYLCS.[1].TPASDPSVWELSRY.[2].SAADDPQL.[1].MKIFWSSLQ 125
gi 462480    60 TPEA.[1].VFGCWRILDTTGPYMLKNTFPELLH.[2].EAPCSPHIWELSRF.[6].KGSLGFSD.[2].LEAMRALAR 130
gi 12230962  61 MSRQ.[1].ICGCARLLPTTDAYLLKDVFAYLCS.[1].TPPSDPSVWELSRY.[2].SAADDPQL.[1].MKIFWSSLQ 125
gi 20140007  59 DADG.[1].ICGCARLLPTTRPYLLKDVFAPLLM.[3].PAPESPGVWELSRF.[7].RARGARPD.[3].RPMLASVVQ 132

gi 13475341 144 FGLANNLTRIVTVTDTRMERILRLATWPLSRIGKPQPVGKTEAVAGFLEISHAS.[26]. 223
query       126 CAWYLGASSVVAVTTTAMERYFVRNGVILQRLGPPQKVKGETLVAISFPAYQER       179
gi 462480   131 YSLQNDIQTLVTVTTVGVEKMMIRAGLDVSRFGPHLKIGIERAVALRIELNAKT.[17]. 201
gi 12230962 126 CAWYLGASSVVAVTTTAMERYFVRNGVILQRLGPPQKVKGETLVAISFPAYQER.[22]. 201
gi 20140007 133 CAAQRGARRLIGVTFVSMVRLFRRIGVRAHHAGPVRCIGGRPVVACWIDIDAST.[18]. 204
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Figure 7. Multiple amino acid sequences of the conserved domains of the Acyl-homoserine lactone of *P. aeruginosa* isolate P14 (AhlS; query) with the top listed homologous proteins from the GenBank using http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi (Marchler-Bauer et al., 2015).The numbers indicate the position within the corresponding proteins: gi 13475341 = conjugation factor synthase traI of nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*, accession code (AC)= NP_106905;gi 462480 = Acyl-homoserine-lactone synthase the autoinducer synthesis protein LasI of *P. aeruginosa*, AC= LASI_PSEAE; gi 12230962 = Acyl-homoserine-lactone synthase the autoinducer synthesis proteinRhlI of *P. aeruginosa*, AC= RHLI_PSEAE; gi 20140007 = Acyl-homoserine-lactone synthase the autoinducer synthesis protein SolI of plant pathogen *Ralstonias olanacearum*, AC= SOLI_RALSO. Amino acids aligned to form position specific scoring matrix (PSSM) where red or blue colors represent degree of conservation with red designating highly conserved. Dashes indicate variation in sequence length among aligned proteins.

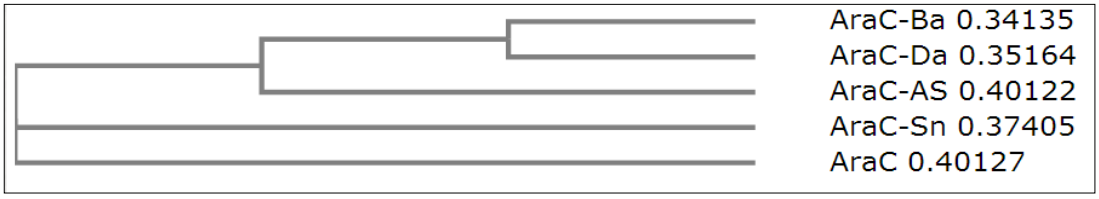

Figure 8. Cladogram of the phylogram analysis of the AraC transcriptional regulator protein of *P. aeruginosa* isolate P14 (AraC query) and its homologous proteins from the GenBank database using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). AraC-AS = transcriptional regulator AraC family, accession code (AC) = Q5LUJ7; AraC-Sn = AraC family transcriptional regulator of *Stackebrandtianassauensis* DSM 44728, AC = YP_003512645; AraC-Ba = AraC family transcriptional regulator of *Burkholderiaambifaria* MC40-6, AC= YP_001815544; AraC-Da = AraC family transcriptional regulator of *Desulfatibacillumalkenivorans* AK-01, AC= YP_002433305.

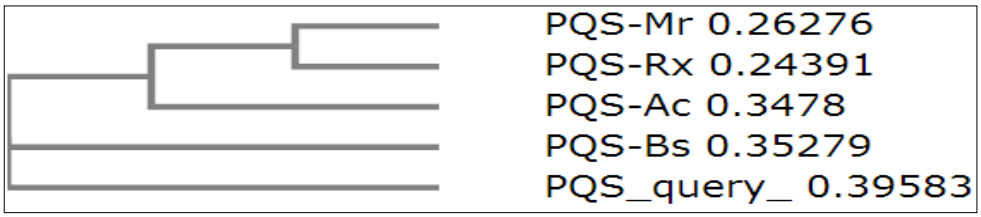

Figure 9. Cladogram of the phylogram analysis of the putative 2-heptyl-3-hydroxy-4(1H)-quinolone synthase of *P. aeruginosa* isolate P14 (PQS, query) and its homologous from the GenBank database using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). PQS-Ac = 2-heptyl-3-hydroxy-4(1H)-quinolone synthase protein of *Acinetobacter* sp. ADP1, accession code (AC) = YP_046465; PQS-Mr = monooxygenase FAD-binding protein of *Meiothermus ruber* DSM 1279, AC = YP_003508322; gi PQS-Rx = FAD-binding FAD dependent oxidoreductase of *Rubrobacter xylanophilus* DSM 9941; AC = YP_644990; PQS-Bs = oxidoreductase of *Bacillus subtilis* subsp. *subtilis*str. 168, Rossmann-fold NAD(P)(+)-binding proteins; cl21454, AC = NP_388604.

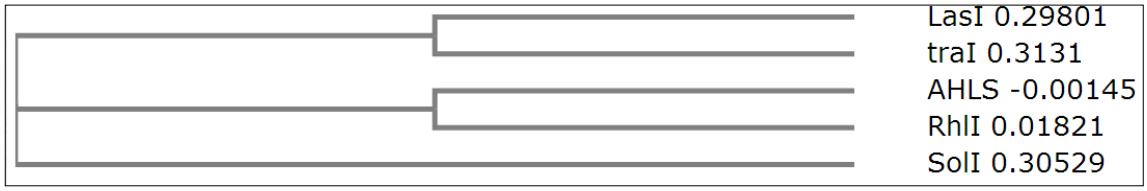

Figure 10. Cladogram of the phylogram analysis of AhlS and its homologous proteins from the GenBank database using ClustalW (http://www.ebi.ac.uk/Tools /msa/clustalw2). LasI =Acyl-homoserine-lactone synthase, an autoinducer synthesis protein of *P. aeruginosa*, AC: LASI_PSEAE; traI = conjugation factor synthase of *Mesorhizobium loti*, AC: NP_106905.1; RhLI = Acyl-homoserine-lactone synthase, an autoinducer synthesis protein of *P. aeruginosa*, AC: RHLI_PSEAE; SolI= acyl-homoserine-lactone synthase, autoinducer synthesis protein *of Ralstonia solanacearum*, AC: SOLI_RALSO. AC= Protein accession code within the GenBank database.

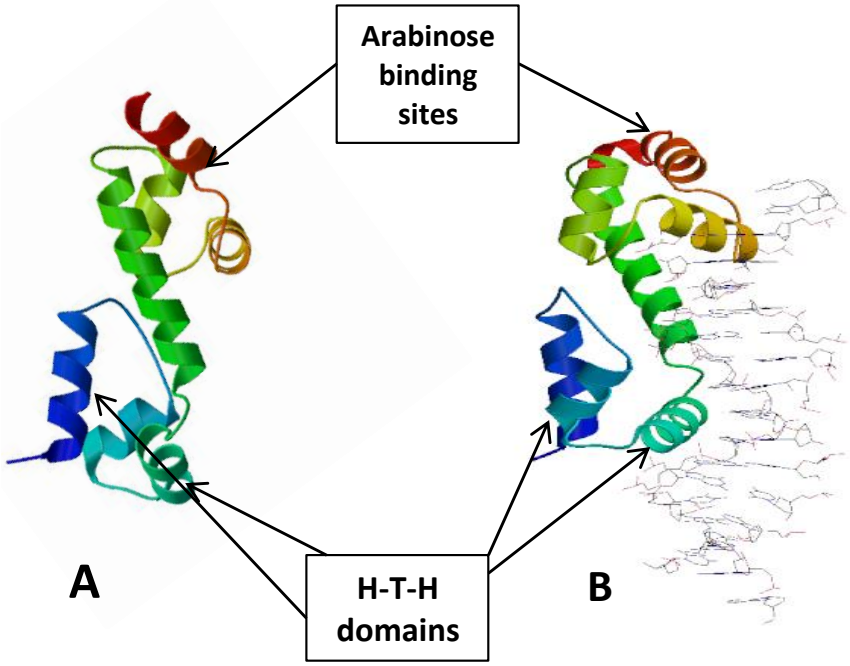

Figure 11. The putative tertiary structure using SWISS-MODEL homology modeling report of: A, putative Arabinose transcription regulatory protein of *P. aeruginosa* isolate P14 (AraC),B,Crystal structure of the DNA-binding domain of AdpA, the global transcriptional factor, in complex with a target DNA, X-RAY DIFFRACTION 2.95 Å (Yao et al., 2013).Arrows indicated both arabinose binding domains (conserved catalytic sites) at the N-terminal and conserved C-terminal helix-turn-helix domains (H-T-H domains).

was predicated using a standard template model of a HTH-type transcriptional activator structure of the DNA-binding domain of AdpA, the global transcriptional factor, in complex with a target DNA, X-RAY DIFFRACTION 2.95 Å. A model of AraC revealed high degree of similarities (QMEAN4 score of -2.99) to the template model as well as conserved N-terminal arabinose binding (catalytic site) and C-terminal Helix-Turn-Helix domains (H-T-H domains). A model for the putative tertiary structure of the 2-heptyl-3-hydroxy-4(1H)-quinolone synthase (PqsS)of *P. aeruginosa* isolate P14 showedhigh degree of similarities with the template, flavin-containing monooxygenase (PhzS) from *P. aeruginosa*. The model showed conserved N-terminal, C-terminal and alpha helices domains (Figure 12). Moreover, the predicated tertiary structure of acyl-homoserine lactone synthase (AhlS) of *P. aeruginosa* isolate P14 was built and showed high degree of conservation at both N-and C-terminals of a template coded N-acyl-homoserine lactone synthase, LasI (Figure 13).

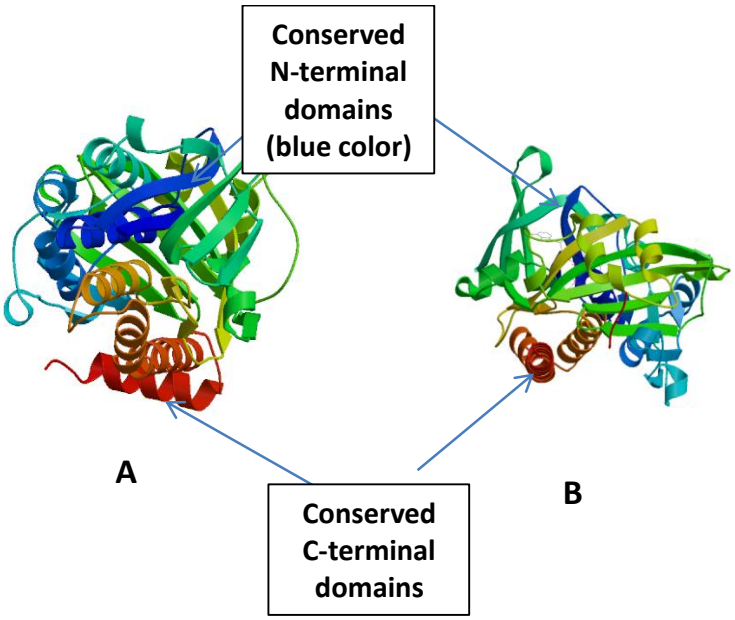

Figure 12. The putative tertiary structure using SWISS-MODEL homology modeling report of: A, putative 2-heptyl-3-hydroxy-4(1H)-quinolone synthase of *P. aeruginosa* isolate P14 (PqsS);B, crystal structure of flavin-containing monooxygenasephzS from *P. aeruginosa(*http://www.uniprot.org/uniprot/Q9HWG9*)* using X-RAY DIFFRACTION 1.90 Å.

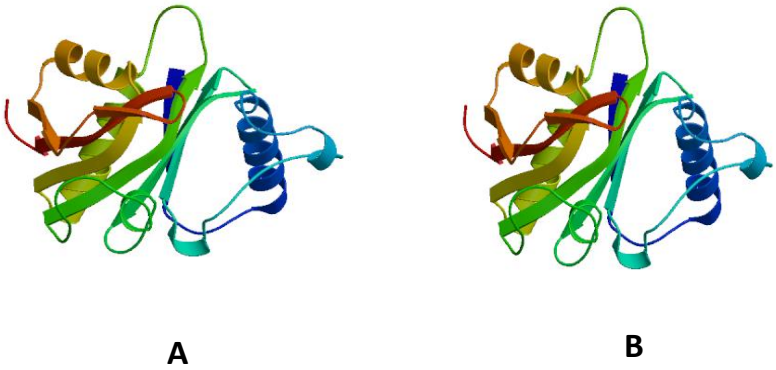

Figure 13. The putative tertiary structure using SWISS-MODEL homology modeling report of: A, putative Acyl-homoserine-lactone synthase, an autoinducer synthesis protein of *P. aeruginosa* isolate P14 (AhlS);B, Crystal structure of the AHL Synthase LasI using X-RAY DIFFRACTION 2.30 Å. (Chung et al., 2011).

DISCUSSION

Limited number of newly discovered antibiotics, spread and rapid emergence of microbial resistance as well as the extreme cost required for isolation of new antimicrobial agents necessitated an urgent need for finding or discovering new approaches for infection control (Wisedchaisri et al., 2014; Hinchliffe et al., 2014; Xu et al., 2014; Shilton, 2015; Zhang et al., 2015). Several studies have recently been conducted to explore microbe-microbe interactions among coexisted organisms in various infectious sites in order to investigate different factors as well as molecules affecting these types of interactions (Jagath et al., 1996; Kadurugamuwa and Beveridge, 1996; Hoffman et al., 2006; Manefield and Whiteley, 2007; Atkinson and Williams; 2009; Biswas et al., 2009; Riedele and Reicht, 2011; Pastar et al., 2013; Fugère et al., 2014; Rüger et

al., 2014). The purpose of studying microbial interactions is to find new molecules that can be later manipulated to be used as therapeutics for the control of the clinically relevant antibiotic-resistant pathogens such as MRSA (Donlan and Costerton, 2002; Martineau et al., 2007).

Therefore, the present study aimed to study the influence of plasmid acquisition on the inhibitory effect of culture supernatantof certain *P. aeruginosa* clinical isolates on the biofilm formation of methicillin resistant *S. aureus*(MRSA) that were co-isolated from pus specimens from infected human wounds. Therefore, various clinical specimens have been collected and investigated for presence of co-existed pathogens. Two models of mixed cultures were selected for the current study: i) *P. aeruginosa* clinical isolate P14 (harbouring no plasmids) co-existing with a methicillin resistant *S. aureus* (MRSA) S14; and ii) *P. aeruginosa* clinical isolate P12 (harbouring plasmids) coexisting with another isolate of MRSA isolate S12. Both mixed cultures were selected for studying the influence of presence or absence of the endogenous plasmids on the inhibitory effect of the culture supernatant of *P. aeruginosa* clinical isolates, the biofilm formation of co-existing MRSA clinical isolates. Results showed the culture supernatant of *P. aeruginosa* without endogenous plasmids exerted a significant reduction effect on the biofilm formation of the co-existed MRSA isolate (about 57% reduction; $P < 0.005$) however, the culture supernatant of *P. aeruginosa* with endogenous plasmids showed an increase of the biofilm formation of the co-existed MRSA isolate (about 5% increase, $P < 0.05$). This inhibitory effect has been confirmed using plasmid transformation experiments and genetic analysis of enzymes previously documented to be involved in the regulation and synthesis of major molecules of quorum sensing of *P. aeruginosa* particularly when coexisting with Gram positive pathogens. Plasmids extracted from *P. aeruginosa* clinical isolate P12 were used to transform competent cells prepared from *P. aeruginosa* clinical isolate P14 according to protocol described by Sambrook and Russell (2001). Results showed that only three smaller plasmid bands out of six had been successfully transformed into *P.aeruginosa* clinical isolate P14 host strain. Reasons for unsuccessful transformation of the other three plasmid bands could be their larger sizes. The newly transformant *P.aeruginosa* clinical isolate P14t was co-cultured with MRSA isolate S14 and the effect of culture supernatant *P. aeruginosa* clinical isolate P14t on the biofilm formation of MRSA isolate S14 have shown about 5% reduction in the biofilm formation. The interpretation of this could be attributed to the decrease in cost fitness of the host strain harbouring plasmids due to energy consumed for replication of the endogenous plasmids (high plasmid copy number) as well as mRNA expression in comparison with the other host strain harbouring no plasmids (Søndergaard et al., 2015). This finding is supported by San Millan et al. (2015) and other investigators who proved that the interaction between horizontally acquired genes create a fitness cost in *P. aeruginosa* (San Millan et al., 2015; Vogwill and MacLean, 2014).

Moreover, the genetic analysis of the genes/enzymespreviously documented to be involved in the regulation and synthesis of major molecules of quorum sensing of *P. aeruginosa* particularly when coexisting with Gram positive pathogens have been studiedto ensure absence of any mutational or conformational change of the nucleotide or amino acid sequences of the respective genes/gene products (Stover et al., 2000). These genes/enzymes include, AraCfamily transcriptional regulator, 2-heptyl-3-hydroxy-4(1H)-quinolone synthase (PqsS) and acyl-homoserine-lactone synthase (AhlS).Primers were designed based on the conserved amino acid sequences of various homologous proteins to the respective enzymes located in the GenBank database. The designed primers were used for target amplification of the target sequences using chromosomal DNA of *P. aeruginosa* isolateP14 as a PCR template. The PCR products obtained were analyzed using agarose gel electrophoresis, purified and sequenced in both forward and reverse directions. The obtained sequence files were assembled using Staden Package (Staden, 1996) into two final consensus sequences of 2.208 kb (harbor a predicted AraC family transcriptional regulator, 1026 bp, 341 aa, accession codes, KT693035;and2-heptyl-3-hydroxy-4(1H)-quinolone synthase, 1149 bp, 382 aa accession codes, KT693034)and 0.54 kb (harbor acyl-homoserine-lactone synthase, 540 bp (partial 3' terminus, 179 aa, accessioncode, KT693033).

Analysis of the conserved domains, phylogenetic analaysis and the tertiary structures revealed that, AraC was a model of AraC transcription regulator with a conserved N-terminal arabinose-binding domain and C-terminal H-T-H motive of the protein family pfam12625 and located upstream of the PqsS(Marchler-Bauer et al., 2015; Aboshanab and Elshafey, 2015). PqsS was a model of putative 2-heptyl-3-hydroxy-4(1H)-quinolone synthase with a conserved domain of a NAD(P)-binding Rossmann-like domain of the protein family, pfam13450. The NADB domain is in many dehydrogenases of metabolic pathways including glycolysis and redox enzymes. NAD binding involves many hydrogen-bonds and van der Waals contacts, in particular H-bonding of residues in a turn between the first strand and the subsequent helix of the Rossmann-fold topology. They also exhibit a consensus binding pattern similar to GXGXXG, in which the first 2 glycines participate in NAD(P)-binding, and the third facilitates close packing of the helix to the beta-strand. They also contain a second domain which is responsible for substrate binding and catalyzing a particular enzymatic reaction (Marchler-Bauer et al., 2015; Hanukoglu, 2015). The AhlS revealed more than 85% similarities in the amino acid sequences with its homologous proteins encoding diverse acyl-

homoserine lactone synthase proteins. AhlS protein has a putative conserved domain of N-acyl-L-homoserine lactone synthetase (accession: COG3916) as well as the auto inducer synthetase of the protein family, pfam00765 (Marchler-Bauer et al., 2015). The tertiary structure of AraC was predicated using a standard template model of a HTH-type transcriptional activator AdpA, the global transcription factor in *Streptomyces griseus* using X-RAY DIFFRACTION 2.00 Å (Yao et al., 2013).. A model of AraC revealed a QMEAN4 score of -2.99 and revealed the structural basis of its tolerant DNA sequence specificity. The model showed a conserved N-terminal arabinose binding (catalytic site) and C-terminal helix-turn-helix domains (Yao et al., 2013). A model for the putative tertiary structure of the 2-heptyl-3-hydroxy-4(1H)-quinolone synthase (PqsS) of *P. aeruginosa* isolate P14 showed more than 80% similarity with crystal structure of *Klebsiellapneumoniae* R204Q HpxOcomplexed with FAD (a QMEAN4 score of -6.21) (Hicks et al., 2013) and to the template, flavin-containing monooxygenase (PhzS) from *P. aeruginosa* (a QMEAN4 score of -7.55). The model showed conserved N-terminal, C-terminal and alpha helices domains. Moreover, the predicated acyl-homoserine lactone synthase (AhlS) of *P. aeruginosa* isolate P14 was built and showed high degree of conservation (a QMEAN4 score of -3.18) at both N-and C-terminals of a template coded N-acyl-homoserine lactone synthase (Chung et al., 2011). In conclusion, plasmid acquisition decreases the inhibitory effect of *P. aeruginosa* on the biofilm formation of MRSA and such effect was not correlated with any deviation in the nucleotide/amino acid sequences of the respective ORFs or protein structures of enzymes that were previously documented to be involved in quorum sensing.

Conflict of Interests

The authors have not declared any conflict of interests.

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