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Aspirin is an efficient inhibitor of quorum sensing, virulence and toxins in *Pseudomonas aeruginosa*

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ABSTRACT

Quorum sensing (QS) plays a vital role in regulation of virulence factors and toxins in *Pseudomonas aeruginosa*, which can cause serious human infections. Therefore, the QS system in *P. aeruginosa* may be an important target for pharmacological intervention. Activity of aspirin on the QS system was assessed using a reporter strain assay and confirmed using RT-PCR to test expression of virulence factors and toxins. In addition, molecular modeling techniques including docking, flexible alignment and surface mapping were also applied to further understand aspirin's potential QS inhibition activity. Aspirin (6 mg/ml) showed significant reduction ($p < 0.01$) of quorum sensing signals in *P. aeruginosa*, including expression of elastase, total proteases, and pyocyanin ($p < 0.01$) without affecting bacterial viability. Aspirin also significantly reduced organism motility and biofilm production ($p < 0.01$) and decreased expression of *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA* and *pqsR* genes by 38, 72, 69, 72, 74 and 43% respectively. Moreover, the expression of *Pseudomonas* toxins *exoS* and *exoY* was reduced by 47 and 55% respectively. The molecular modeling analysis suggests the QS inhibitory action of aspirin occurs through interaction of aspirin's aryl group and Tyr-88 of the LasR receptor, by strong π–π stacking interactions, which associated with a conformational change of the receptor–aspirin complex. The inhibitory effect of aspirin on virulence factors was specific to *P. aeruginosa* as aspirin at sub-MIC did not affect the biofilm or motility of *Escherichia coli*.

To summarize, the collective data demonstrate that low concentrations of aspirin inhibit quorum sensing of *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is an opportunistic and nosocomial cause of human disease, especially in immunocompromised patients. It can cause cystic fibrosis, urinary tract infections, burn infections and many other conditions. Furthermore, it is associated with a high incidence of antibiotic resistance and biofilm formation [1].

Existing antibiotics are losing the battle against *P. aeruginosa* due to bacterial resistance, leading scientists to search for new approaches for managing infections. The *P. aeruginosa* quorum sensing (QS) system is a key regulator of biofilm development [2] antibiotic resistance [3] and other pathogenic functions including expression of virulence factors [4]. Quorum sensing inhibitors (QSIs) diminish *P. aeruginosa* pathogenesis [5] and attenuate microbial virulence, allowing the host immune system to clear the infection without concern for antibiotic resistance [1].

P. aeruginosa QS genes function in a hierarchical manner with the prominent *lasI/R* system [6] controlling the activity of *rhlI/R* circuit [7]. The las system comprises LasI, which is governed by the transcriptional regulator LasR that regulates the expression of protease, exotoxin A, elastase, and its autoinducer synthetase (lasI). *P. aeruginosa* produces two discrete acyl homoserine lactone (AHL) molecules: *N*-(3-oxododecanoyl)-L-homoserine lactone (3-OH-C_{12}-HSL) and *N*-butanoyl-L-homoserine lactone (C_4-HSL). These molecules are natural ligands for lasI/R, and rhlI/R and activate virulence response upon binding [8]. Furthermore, the AHL signaling system

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triggers a third *P. aeruginosa* quinolone signaling system (PQS) by producing, the signal molecule, 2-heptyl-3-hydroxy-4(1*H*)-quinolone [9]. This system regulates the expression of virulence factors, bacterial motility and biofilm formation [8].

Some natural and chemically synthesized compounds have been reported to have QSI activity. The most common natural QSIs include halogenated furanone compounds from marine algae *Delisea pulchra* [10] and patulin and penicillic acid from *Penicillium* species [11]. Other known inhibitors include ajoene, a sulfur-rich molecule from garlic [12] and ellagic acid derivatives from *Terminalia chebula* Retz [13]. Chemically synthesized QSIs include AHL analogs such as phenylpropionyl homoserine lactones and phenyloxyacetyl homoserine lactones [14]. A series of cyclopentanols [15], halogenated furanones [16] and other furanone derivatives also have QSI activity [17]. Unfortunately, toxicity of these compounds limits their use in mammalian cells [18]. Other studies have evaluated antibiotics for QSI activity in *P. aeruginosa* using sublethal concentrations [19]; these included ceftazidime, ciprofloxacin and macrolides such as azithromycin [20].

The ability of known drugs to inhibit bacterial quorum sensing has led to exploration of other compounds for potential use in various medical applications. Here we investigate aspirin for potential QSI activity. Aspirin is widely used as antipyretic, anti inflammatory and thrombolytic agent. Our study explored various concentrations of aspirin for QSI activity, looking particularly at virulence factors of *P. aeruginosa* including elastase, total protease, pyocyanin and toxins. In addition, we tested aspirin for an effect on biofilm formation and motility. To further understand the QSI activity of aspirin at a molecular level, we carried out molecular modeling including docking, flexible alignment and surface mapping to investigate possible interaction of aspirin with the LasR receptor.

2. Materials and methods

2.1. Bacterial strains, growth media and conditions

P. aeruginosa PAO1 was used as the wild type strain for the assay of QSI activity of aspirin. Reporter strains; *P. aeruginosa* pME3846 (*rhlI-lacZ*; Tc^r) [21] and *E. coli* MG4/pKDT17 (*lasB*::*lacZ plac-lasR* Ap^r) [6] were used for the assay of *rhlI* and *lasI/R* respectively. The QS deficient *P. aeruginosa* PAO-JP2 (△*lasI*::Tn10, Tc^r; △*rhlI*::Tn501-2, Hg^r) double mutant was used as negative control [22]. All cultures were propagated using Luria Bertani medium (LB broth; tryptone 1%, yeast extract 0.5%, and NaCl 1.0%) at 37 °C. *Escherichia coli* K-12 was propagated in LB media at 37 °C.

2.2. Determination of growth inhibition activity of aspirin

The antimicrobial activity of aspirin was determined using the broth microdilution method [23]. Aspirin (400 mg/ml, pH 7.2) was used for preparing 2-fold serial dilutions in LB broth to 400, 200, 100, 50, 25, 12.5, 6, and 3 mg/ml. Diluted aspirin was inoculated with 0.1 ml PAO1 culture containing 5×10^6 CFU/ml and incubated at 37 °C for 24 h. Minimal inhibitory concentration (MIC) was calculated as the lowest concentration of aspirin that inhibited visible growth of the organism. The number of viable PAO1 colonies treated with subinhibitory concentrations (sub-MIC) of aspirin (1/4 MIC) were counted and compared to the count of untreated PAO1 using the pour plate method [24].

Furthermore, the growth of both untreated *P. aeruginosa* and *P. aeruginosa* treated with 1/4 MIC of aspirin was monitored. LB with aspirin (1/4 MIC) and control without aspirin were inoculated with overnight culture of PAO1 and were incubated at 37 °C for 24 h. Samples were taken every hour for measuring OD 600 nm for aspirin treated and untreated cultures [25].

2.3. Inhibition of QS signal molecules

Overnight cultures of *P. aeruginosa* PAO1 (0.5 ml) was inoculated into 5 ml LB broth containing sub-MICs of aspirin (1/4, 1/8 and 1/20 of its MIC). Cultures were incubated with shaking (150 rpm) at 37 °C for 16–18 h. Untreated PAO1 cultures (positive control) and PAO-JP2 (negative control) were also propagated under the same conditions [26]. Cultures were then centrifuged at 6000× *g* for 15 min at 4 °C. Supernatants were filtered twice using a 0.45 μm syringe filter. Cell free filtrates were separated, stored at −20 °C, and used for assay of AHLs and virulence factors [27].

3-OH-C_{12}-HSL and C_4-HSL were assessed in treated and untreated supernatants of *P. aeruginosa* PAO1 using *E. coli* MG4 (pKDT17) for 3-OH-C_{12}-HSL and *P. aeruginosa* (pME3846) for C_4-HSL [6,21]. Overnight cultures of the reporter strains were diluted to 0.1 OD 600 nm and 1 ml of *P. aeruginosa* supernatant was mixed with 0.5 ml of each reporter strain. Cells were propagated for 2 h then pelleted and measured for β-galactosidase activity using Miller assay [28]. All assays were performed in triplicate.

2.4. Effect of aspirin on Pseudomonas virulence factors

The effect of aspirin on the virulence factors of *P. aeruginosa* PAO1 was performed using previously prepared culture supernatant. LasB elastase testing was carried out using Elastin Congo Red (ECR, Sigma Chemicals, St. Louis, USA) (10 mg/ml) [29]. PAO1 supernatant (0.5 ml) was mixed with 10 mg of ECR and 0.5 ml of ECR buffer (100 mM Tris–HCl, pH 7.5). The mixture was then incubated for 6 h at 37 °C with shaking. Following centrifugation to remove insoluble ECR, OD 495 nm was measured.

Total proteolytic activity was determined using the modified skim milk assay [19]. Culture supernatants of PAO1 (0.5 ml) were incubated with 1 ml skim milk (1.25%) at 37 °C for 30 min and turbidity was measured at OD 600 nm.

The hemolysin test was carried out by incubating 0.7 ml of fresh sheep red blood cells (2%) with 0.5 ml of bacterial supernatant at 37 °C for 2 h. Hemoglobin release was measured at OD 540 nm. Controls were RBCs in LB broth only for a negative control, and total RBCs lysed with SDS (0.1%) as a positive control [30].

The pyocyanin assay was performed using King A media (peptone 2%, K_2SO_4 1%, and $MgCl_2$ 0.14%) with and without aspirin. Medium was inoculated with PAO1 and grown at 37 °C for 48 h with shaking at 200 rpm. The OD 520 nm of pyocyanin was measured and concentration determined by the formula concentration (μg/ml) equals OD 520 nm × 17.072 [31]. All assays were performed in triplicate.

2.5. Biofilm formation

Biofilms were developed in a 96 well polystyrene microtiter plate. *P. aeruginosa* cultures (0.1 ml) were incubated for 24 h at 37 °C with different concentrations of aspirin (1/4, 1/8 and 1/20 MIC). Free planktonic cells were removed and biofilm was washed with PBS (10 mM Na_2PO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.0). The biofilm was fixed with 150 μl of methanol for 15 min, air dried and stained with 200 μl of 0.5% (w/v) crystal violet for 15 min. The plate was washed with PBS three times to remove excess stain. Crystal violet was dissolved using 200 μl of 33% (v/v) glacial acetic acid and the absorbance was measured at OD 495 nm [32]. Reported values are the mean of three measurements.

2.6. Motility assays

Plates were inoculated with diluted overnight PAO1 culture grown in the presence and absence of different aspirin concentrations. The swimming plates (1% tryptone, 0.5% NaCl, and 0.5% agar) were inoculated on the surface with 5 μl culture and incubated at 37 °C for 24 h [33], whereas the twitching plates were inoculated with 2 μl of PAO1 at the bottom of 1% LB agar plates and incubated at 37 °C for at least 48 h [34].

2.7. Effect of aspirin on virulence of other gram negative bacteria

The influence of sub-MIC of aspirin on *E. coli* K-12 was also performed. The effect of aspirin on biofilm formation and bacterial motility was carried out as previously described [32,33].

2.8. Expression of QS genes

P. aeruginosa PAO1 was treated with aspirin (6 mg/ml) and total RNA was extracted by TRIzol reagent (Sigma Chemicals, St. Louis, USA). Complementary DNA synthesis was prepared using QuantiTect Reverse Transcription kit (QIAGEN, Germany). RT-PCR was done using 5× FIREPol Eva Green, qPCR Mix, ROX Dye (Solis BioDyne, Tartu, Estonia) using primers described in Table 1, below. RT-PCR was performed using a Rotor Gene Q thermocycler (QIAGEN, Hilden, Germany) programmed as follows: 95 °C for 15 min, then (denaturation at 95 °C for 15 s, annealing at 55–60 °C for 30 s and extension at 72 °C for 1 min) × 40 cycles. Expression of the target genes was normalized to the expression of reference gene *ropD*. The level of gene expression in both treated and untreated samples was calculated relative to the standard sample, untreated PAO1. Gene expression of the double mutant PAO-JP2 was also performed. All measurements were performed in triplicate.

2.9. Statistical analysis

For statistical analyses, the Excel data analysis package was used to calculate mean and standard deviation of the mean. Data was analyzed using the GraphPad Instate software package (version 3.05) according to the Tukey–Kramer multiple-comparison test at a $p < 0.05$ or $p < 0.01$ for significance. All results were calculated from the mean of three separate experiments. The results were expressed as mean ± standard deviation.

Table 1
PCR primers utilized in RT-PCR.

Gene name	Type	Primer sequence	Annealing temp.	Amplicon size (bp)
ropD	Fw	5′–CGAACTGCTTGCCGACTT–3′	56 °C	131
PA0576	Rev	5′–GCGAGAGCCTCAAGGATAC–3′		
lasI	Fw	5′–CGCACATCTGGGAACTCA–3′	56 °C	176
PA1432	Rev	5′–CGGCACGGATCATCATCT–3′		
lasR	Fw	5′–CTGTGGATGCTCAAGGACTAC–3′	55 °C	133
PA1430	Rev	5′–AACTGGTCTTGCCGATGG–3′		
rhlI	Fw	5′–GTAGCGGGTTTGCGGATG–3′	58 °C	101
PA3476	Rev	5′–CGGCATCAGGTCTTCATCG–3′		
rhlR	Fw	5′–GCCAGCGTCTTGTTCGG–3′	58 °C	160
PA3477	Rev	5′–CGGTCTGCCTGAGCCATC–3′		
pqsA	Fw	5′–GACCGGCTGTATTCGATTC–3′	55 °C	74
PA0996	Rev	5′–GCTGAACCAGGGAAAGAAC–3′		
pqsR	Fw	5′–CTGATCTGCCGGTAATTGG–3′	55 °C	142
PA0964	Rev	5′–ATCGACGAGGAACTGAAGA–3′		
exoS	Fw	5′–CCATCACTTCGGCGTCACT–3′	58 °C	129
PA3841	Rev	5′–GAGAGCGAGGTCAGCAGAG–3′		
exoY	Fw	5′–TGCCATAGAATCCGTCCTC–3′	55 °C	145
PA2191	Rev	5′–GATGACCGCCGATTATGAC–3′		

2.10. Modeling methodology

The crystallographic structure of the *P. aeruginosa* LasR ligand binding domain with its natural ligand *N*-(3-oxododecanoyl)-L-homoserine lactone (3-OH-C_{12}-HSL; Protein Data Bank [PDB] identification [ID]: 2UV0) [35] was used in our modeling study. The LasR structure contains four monomers of the ligand binding domain each in a complex with the 3-OH-C_{12}-HSL ligand. The E monomer in a complex with 3-OH-C_{12}-HSL was chosen for analysis via the 'Molecular Operating Environment' MOE workspace (Chemical Computing Group Inc software, Core 2 duo 2.3 GHz workstation Montreal, Canada). Water was excluded from the workspace. The three-dimensional structures of aspirin and C30 furanone (the standard QSI) in their neutral forms were constructed using the MOE. The lowest energy conformer of aspirin (global-minima) was docked into the *P. aeruginosa* LasR ligand binding domain bound to its natural signal 3-OH-C_{12}-HSL. All the hydrogens were added and the enzyme structure was subjected to a refinement protocol in which constraints on the enzyme were gradually minimized with the molecular mechanical force field 'AMBER' until the root mean square gradient was 0.01 kcal/mol Å. The energy-minimized structure was next used for molecular dynamics studies. For each ligand examined, energy minimizations were performed using 1000 steps of the steepest descent, followed by conjugate gradient minimization to a root mean square energy gradient of 0.01 kcal/mol Å. The active site of the enzyme was defined using a radius of 10.0 Å around natural ligand [36,37].

2.11. Flexible alignment

The investigated compounds aspirin, natural ligand of LasR (3-OH-C_{12}-HSL) and LasR inhibitor (C30 furanone) were subjected to a flexible alignment experiment using software (MOE of Chemical Computing Group Inc., on a Core 2 duo 2.3 GHz workstation) [38,39]. The molecules were built using the builder module of MOE. Their geometry was optimized by using the MMFF94 force field followed by a flexible alignment using a systematic conformational search. The lowest energy aligned conformation(s) were identified [40].

2.12. Surface mapping

Aspirin and LasR inhibitor (C30 furanone) were modeled using the builder module of MOE. Their geometry was optimized using the MMFF94 force field followed by computing surface mapping.

3. Results

3.1. Growth inhibitory activity of aspirin

First, the minimal inhibitory concentration of aspirin against *P. aeruginosa* PAO1 was determined as 25 mg/ml. Next, subinhibitory concentrations of aspirin corresponding to 1/4, 1/8 and 1/20 MIC (6, 3 and 1 mg/ml of aspirin respectively) were used to assess QSI activity and inhibition of virulence factors. Viability of *P. aeruginosa* PAO1 treated with sub-MIC of aspirin was similar to viability of untreated PAO1. For example, at 1/4 MIC bacterial counts of 155 × 10^6 CFU/ml in treated cultures compared to counts of untreated cultures (161 × 10^6 CFU/ml). In addition, both treated and untreated PAO1 reached stationary phase after 8 h incubation at 37 °C (Fig. 1A), indicating no effect on growth rate.

3.2. Inhibition of Las/Rhl signaling

Aspirin was tested for QSI activity targeted on the *las and rhl* genes using a reporter strain assay. Aspirin (3 and 6 mg/ml) showed significant reduction of QS signals C_4-HSL and 3-OH-C_{12}-HSL. The level of 3-OH-C_{12}-HSL decreased in a dose-dependent manner; 63% reduction with 1/4 MIC ($p < 0.01$) and 53% reduction with 1/8 MIC of aspirin ($p < 0.05$). A lower concentration of aspirin (1/20 MIC) showed no significant effect (38% reduction, Fig. 1B).

The level of C_4-HSL produced was also significantly lowered, by 64%, 66% and 76% at 1/20, 1/8 and 1/4 MICs of aspirin respectively ($p < 0.01$) (Fig. 1C). As a negative control, strain PAO-JP2, which is double mutant for *lasI* and *rhlI* also showed low activity for 3-OH-C_{12}-HSL and C_4-HSL. Aspirin produced no effect on β-galactosidase activity of reporter strains pME3846 or MG4.

3.3. Influence of aspirin on virulence factors

We next tested the effect of aspirin on the production of QS-controlled virulence factors including total protease, elastase, hemolysin, and pyocyanin. In addition, we tested biofilm formation. Treating *P. aeruginosa* PAO1 with 1/4 (6 mg/ml) or 1/8 MIC (3 mg/ml) of aspirin caused significant reduction in the level of all virulence factors tested (Fig. 2A). At 6 mg/ml, aspirin reduced elastase, protease, hemolysin and pyocyanin to the level of PAO-JP2. Aspirin also significantly decreased protease activity at 1/8 and 1/4 MIC by 58 and 82% ($p < 0.01$), although 1/20 MIC did not cause a significant change (30%) In contrast, the level of hemolysin was significantly decreased with all tested concentrations of aspirin; even 1/20 aspirin eliminating 63% of hemolytic activity. Aspirin also displayed significant reduction in elastase activity, by 60 and 70% respectively at 1/8 and 1/4 MIC ($p < 0.01$), although there was no significant effect at 1/20 MIC. Furthermore, aspirin significantly affected biofilm formation at 1/4 MIC (50% reduction) with $p < 0.01$with no significant effect at 1/8 and 1/20 MIC. In addition, aspirin decreased pyocyanin at all tested concentrations with 78% reduction with 1/4 MIC of aspirin (Fig. 2A).

3.4. Aspirin effect on cell motility

Influence of sub-MICs of aspirin on the motility functions twitching and swimming was measured by inoculating motility plates with diluted cultures of PAO1 (OD 600 nm 0.4–0.5). Complete arrest of twitching and swimming was obtained with aspirin at 1/4 MIC and lower concentrations of aspirin still caused significant reduction of twitching (45%, $p < 0.05$) and some reduction of swimming (34%). Aspirin at 1/4 MIC produced an effect similar to the double mutant strain PAO-JP2 as shown in Fig. 2B.

3.5. Effect of aspirin on virulence of other gram negative bacteria

At sub-MIC aspirin had no significant effect on biofilm formation on *E. coli* K-12. *E. coli* cultures treated with aspirin developed a similar amount of biofilm as untreated *E. coli*. Furthermore, 1/4 sub-MIC concentrations of aspirin decreased *E. coli* motility by 30% which was not a significant value.

3.6. Expression of QS-regulated genes

Relative expression of QS regulatory genes was tested on aspirin-treated PAO1 and untreated cultures. The standard curve of the housekeeping gene *ropD* and of all expressed genes including *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA* and *pqsR*, showed R^2 values 0.99–0.97. Furthermore, standards and samples of PAO1 used for each gene expression test had the same melting profile and formation of pure amplicons indicated the specificity of the assay. Relative expression levels of QS-regulated genes were analyzed using the $2^{-\Delta\Delta Ct}$ method [41]. Aspirin at 1/4 MIC significantly repressed the expression of *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA* and *pqsR* by 38, 72, 69, 72, 74 and 43% respectively (Fig. 3).

3.7. Real-time PCR analysis of P. aeruginosa exotoxins

Relative expression of *exoY* and *exoS* was measured from calculated *Ct* values and standard curves. They showed the same melting profiles with no primer dimer formation. RT-PCR data revealed that aspirin (6 mg/ml) caused significant reduction in the expression levels of *exoS* and *exoY*, 47% and 55% respectively, relative to untreated PAO1.

3.8. Molecular docking study between aspirin and LasR

Aspirin lies completely the active site of the LasR legend. As detected from 3D dual binding, hydrogen bonding contributed significantly to aspirin binding to the LasR protein. Specific residues interacting with aspirin included Thr-115 (17% and 19%), Ser-129 (44%) and Thr-75 (88%) (Fig. 4A). Importantly, Tyr-88 seemed to be crucial for aspirin binding and therefore suggested the inhibition of LasR activity. The Tyr-88 aromatic ring was involved in π–π stacking interactions with aspirin and induced a conformational

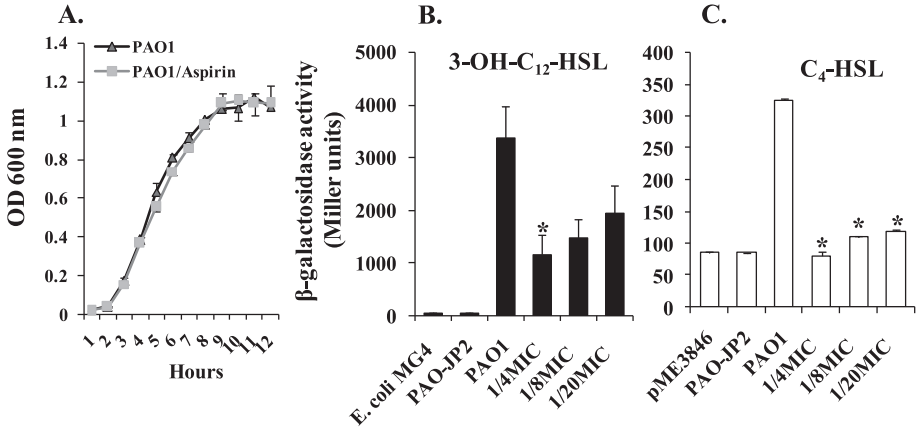

Fig. 1. Effect of sub-MICs of aspirin on the quorum sensing signals; Growth curve of PAO1 in the presence and absence of 1/4 MIC of aspirin (A). Effect of sub-MICs (1/4, 1/8 and 1/20) of aspirin on the levels of 3-OH-C_{12}-HSL (B) and C_4-HSL (C) in PAO1 as detected using reporter strain assay compared to control untreated PAO1. Aspirin (1/4 MIC) caused significant reduction in 3-OH-C_{12}-HSL signal $p < 0.01$. All used sub-MICs (1/4, 1/8, and 1/20) of aspirin caused significant reduction of C_4-HSL (*significant $p < 0.01$).

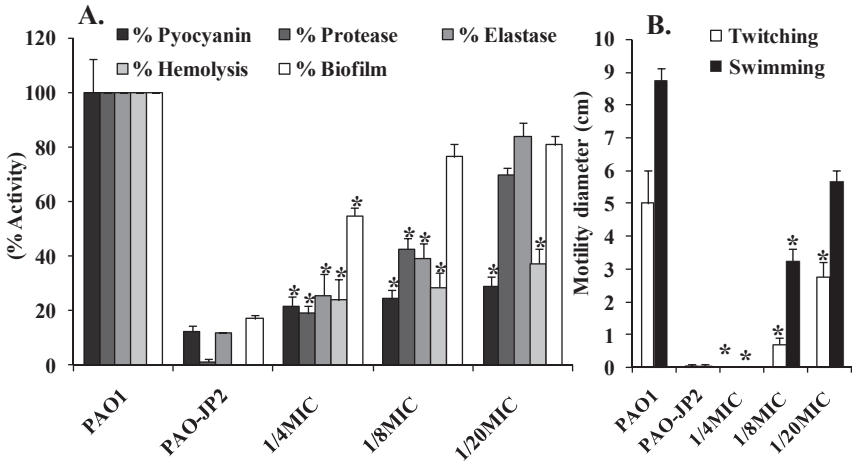

Fig. 2. Influence of aspirin on virulence factors of PAO1. A. Aspirin (1/4 and 1/8 MICs) caused significant elimination in all tested virulence factors pyocyanin, protease, elastase, hemolysin and biofilm compared to control untreated PAO1, but except for biofilm did not significantly reduced by 1/8 MIC of aspirin. B. bacterial motilities twitching and swimming were also inhibited by 1/4 and 1/8 MIC of aspirin (*significant $p < 0.01$).

change that may be responsible for aspirin's LasR antagonist activity. The best fit docking pose of aspirin had S score of −12.0213 as shown in Fig. 4B.

3.9. Flexible alignment structural analysis

Ligand-based active site alignment is a widely adopted technique for the analysis of protein–ligand complexes. Comparative flexible alignment analysis between aspirin and both the natural ligand of LasR (3-OH-C_{12}-HSL) from one side and the LasR inhibitor (C30 furanone) from other side was conducted automatically using the MOE system. Two hundred conformers of each compound were generated and minimized using a distance-dependant dielectric model. The top scoring alignments having least strain energy are shown in Fig. 4C. There was good alignment between aspirin and C30 furanone, consistent with aspirin's QSI activity on LasR. In contrast, Fig. 4D demonstrates that there are different alignment profiles between aspirin and the natural LasR agonist (3-OH-C_{12}-HSL). As for the molecular docking analysis, flexible alignment modeling was consistent with the hypothesis that a conformational change occurs when aspirin binds the LasR enzyme.

3.10. Surface mapping analysis

Another structural study, hydrophobic surface mapping, was performed on LasR complexed with aspirin and the most active known LasR inhibitor, C30 furanone. Fig. 4 E, F shows a similar distribution of hydrophilic (hydrogen bonding) and hydrophobic regions with aspirin and C30 furanone. Thus both inhibitors could have similar interactions with key amino acid residues inside the enzyme active pocket.

4. Discussion

Inhibition of QS is a potential method to control microbial infections. The influence of various natural and synthetic compounds on QS in *P. aeruginosa* has been investigated [16,17,26,42]. *P. aeruginosa* QS is composed of *las*, *rhl* and *pqs* circuits under control of lasI/R system [5,8]. Hence, inhibition of *lasR* will be accompanied by loss of downstream QS signals. At concentration below the MIC, aspirin showed significant inhibition of both quorum sensing signals: 3-OH-C_{12}-HSL and C_4-HSL (Fig. 1). However, growth and viability of *P. aeruginosa* PAO1 were not reduced,

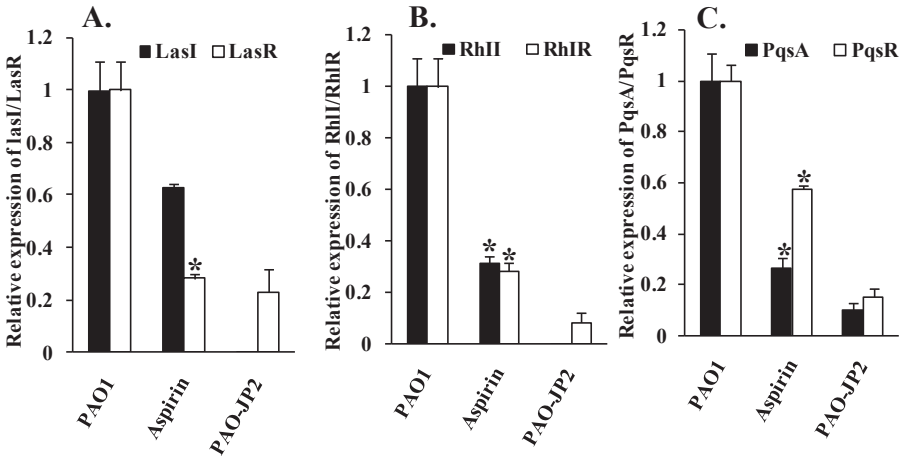

Fig. 3. Aspirin inhibited QS regulatory genes of *P. aeruginosa* PAO1; relative expression of aspirin treated PAO1. A. *lasI*/*lasR*, B. *rhlI*/*rhlR* and C. *pqsA*/*pqsR*. Aspirin 1/4 MIC caused 1.6, 3.5, 3.2, 3.5, 3.8 and 1.8 fold decrease in the expression of *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA* and *pqsR* respectively compared to expression of genes in untreated PAO1 (*significant $p < 0.01$).

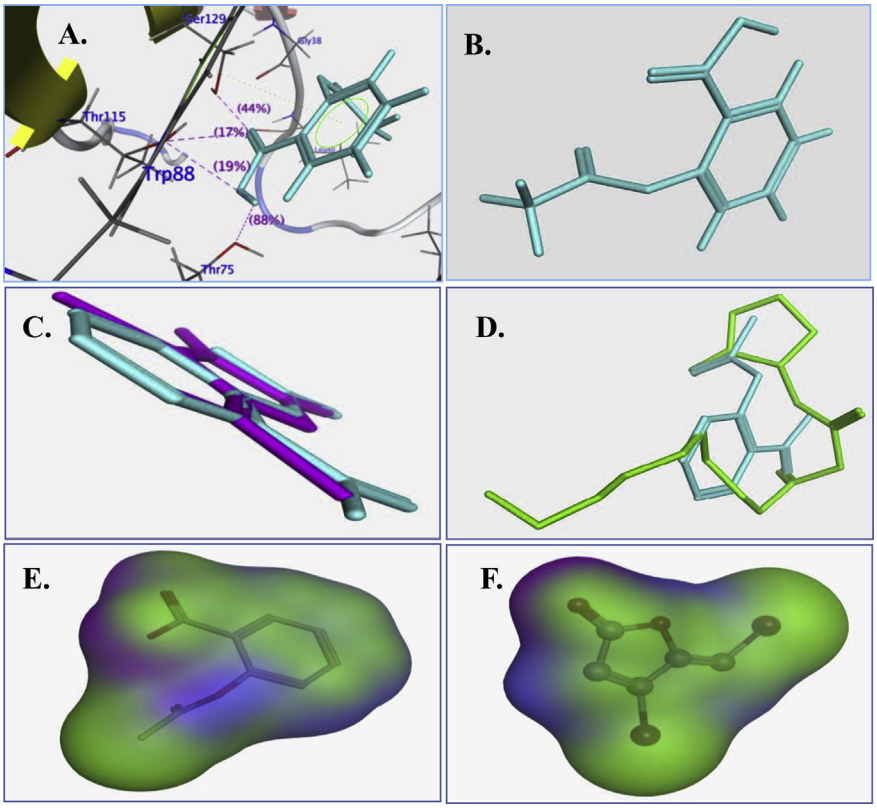

Fig. 4. Modeling analysis of aspirin interaction with Las R; A. 3D Interaction of aspirin (cyan) with the active site model of LasR. B. best docking pose of aspirin. C. Flexible alignment of aspirin and most potent furanone LasR inhibitor C30 furanone (pink). D. Flexible alignment of aspirin (cyan) and natural ligand of LasR enzyme *N*-(3-oxododecanoyl)-L-homoserine lactone (green). E. Surface map for aspirin in pocket side. F. Surface map for C30 furanone in pocket side. Pink, hydrogen bond, blue: mild polar, green hydrophobic. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proving that aspirin's effect was achieved through inhibition of QS not by killing of cells (Fig. 1A). Other studies have also demonstrated that QS inhibitory effect in *P. aeruginosa* and *Acinetobacter baumannii* is achieved through reduction in the level of HSL signals not bacterial growth [19,43–45].

The activity of aspirin against the *las* and *rhl* systems led us to investigate its effects on *Pseudomonas* virulence factor production. *P. aeruginosa* produces hydrolytic enzymes elastase, protease and hemolysin that degrade tissue components and interfere with host defense mechanisms [46]. Aspirin at sub-MICs caused significant reduction ($p < 0.01$) in elastase, hemolysin, protease and pyocyanin activities, to the level of the mutant strain PAO-JP2. Furthermore, the percentage reduction in virulence factors was consistent with the level of reduction in QS signaling molecules (Fig. 2A).

Biofilm development is associated with antibacterial resistance and can contribute to severe infections [3] and the QS system participates in control of biofilm formation. PAO1 biofilm production was significantly reduced with 6 mg/ml aspirin ($p < 0.01$), likely via its effect on QS. QS is known to regulate several factors involved in biofilm production, including rhamnolipids and siderophores [2,47]. In addition, *P. aeruginosa* motility plays a vital role in creation of biofilms by assisting in initial attachment, biofilm formation and bacterial propagation through burns and wounds [48]. In our study, sub-MIC aspirin inhibited both twitching and swimming motility (Fig. 2B), consistent with reports that azithromycin [25], and salicylic acid [42] inhibit swimming motility in *P. aeruginosa*.

The inhibitory effect of aspirin on virulence factors was specific for *P. aeruginosa* as sub-MICs of aspirin did not affect motility or biofilm production in *E. coli* K-12, likely because the *E. coli* QS circuit differs from that of *P. aeruginosa*. Depending on the function of the LuxS gene, *E. coli* produces a signal molecule termed AI-2 [49]. *E. coli* mutants lacking LuxS show lower expression of flagellin and have reduced motility compared with the wild-type [50].

Measurement of QS-regulatory gene expression (*lasI*, *lasR*, *rhlI*, *rhlR*, *pqsR* and *pqsA*) indicated that aspirin can inhibit QS at the transcriptional level (Fig. 3). Aspirin appeared to compete with 3-OH-C_{12}-HSL binding to LasR because it down regulated the *lasR* gene by 72% while causing just 38% inhibition of *lasI* expression (Fig. 3A). In addition, aspirin significantly repressed the C_4-HSL synthase genes *rhlI* and *rhlR* by approximately the same level as *lasR* (Fig. 3B).

Aspirin caused down regulation of the *pqsA* gene by 75%, about the same level as *lasR* (Fig. 3C). The production and activity of PQS was dependent on LasR and RhlR [9]. Yang et al. [51], reported that salicylic acid, the primary metabolite of aspirin, is a potent inhibitor of *pqs*-dependent signaling which may account for its QSI effect. Salicylic acid down regulates *rhlR* and *lasA* in *P. aeruginosa* PA14 [52] with subsequent inhibition of pyocyanin, protease, and elastase activities [4,42,51]. In our study, the decrease in the expression level of *lasR* was consistent with reduction in all virulence factors controlled by *lasI*/*R* (elastase and protease) and *pqs*/*rhl* (biofilm and pyocyanin). *P. aeruginosa* also produces exoenzymes S, T, U, and Y that can damage host cellular machinery leading to cell death [53,54]. Our data revealed that aspirin significantly reduced expression of *exoS* and *exoY* genes. Likewise, genes *exoT*, *exsB*, and *exsC*, which are involved in toxin secretion were significantly repressed by salicylic acid treatment at sub-inhibitory concentrations as previously reported [52].

Using a three dimensional molecular modeling system, the antagonist activity of aspirin could be attributed to interaction between its aryl group and the aromatic amino acid Tyr-88 of the LasR receptor via a strong π–π stacking interactions (Fig. 4A). Also, QSI could be attributed to a change in conformation of the aspirin–receptor complex which gave a surface map very close to that of the complex with the natural inhibitor, C30 furanone. Our modeling study may explain QSI activities of aspirin.

5. Conclusion

This work extends the functions of aspirin to include active inhibition of bacterial virulence by significantly inhibiting at least five of *P. aeruginosa* functions including: (1) quorum sensing; (2) virulence factor production; (3) biofilm formation; (4) toxin production and (5) diverse adhesive factor production. Our modeling study provides a detailed molecular model to aid the interpretation of QSI activity of aspirin. Hydrophobic mapping and conformational analysis show similar interactions between aspirin and the natural inhibitor C30 furanone with the LasR ligand binding site. Because aspirin is a well understood over-the counter drug and because QSI activity takes place at safe concentrations, it may be useful to investigate its *in vivo* activity in treatment of *P. aeruginosa* infections. Because its target action is on QS, aspirin is likely to generate less evolutionary pressure for resistance than traditional antibiotics, thus avoiding a limitation of antibiotic therapy. Future animal and/or clinical studies may yield interesting or even exciting results.

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