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Original Article

Deficient *Pseudomonas aeruginosa* in MlaA/VacJ outer membrane lipoprotein shows decrease in rhamnolipids secretion, motility, and biofilm formation, and increase in fluoroquinolones susceptibility and innate immune response

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ABSTRACT

Pseudomonas aeruginosa, a Gram-negative bacterium that causes severe hospital acquired infections poses threat by its ability for adaptation to various growth modes and environmental conditions and by its intrinsic resistance to antibiotics. The latter is mainly due to the outer membrane (OM) asymmetry which is maintained by the Mla pathway resulting in the retrograde transport of glycerophospholipids from the OM to the inner membrane. It comprises six Mla proteins, including MlaA, an OM lipoprotein involved in the removal of glycerophospholipids mislocalized at the outer leaflet of OM.

To investigate the role of *P. aeruginosa* OM asymmetry especially MlaA, this study investigated the effect of *mlaA* deletion on (i) the susceptibility to antibiotics, (ii) the secretion of virulence factors, the motility, biofilm formation, and (iii) the inflammatory response.

mlaA deletion in *P. aeruginosa* ATCC27853 results in phenotypic changes including, an increase in fluoroquinolones susceptibility and in PQS (*Pseudomonas* Quinolone Signal) and TNF-α release and a decrease in rhamnolipids secretion, motility and biofilm formation.

Investigating how the *mlaA* knockout impacts on antibiotic susceptibility, bacterial virulence and innate immune response will help to elucidate the biological significance of the Mla system and contribute to the understanding of MlaA in *P. aeruginosa* OM asymmetry.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that causes severe hospital-acquired infections [1]. It is the leading cause behind the morbidity and mortality of cystic fibrosis patients and immunocompromised patients [2]. Central to *P. aeruginosa's* success as an opportunistic pathogen is the genetic plasticity and the great metabolic capabilities provided by its large genome.

One hallmark of the *Pseudomonas* species is their high intrinsic level of resistance to antibiotics. The outer membrane (OM) asymmetry with lipopolysaccharides (LPS) at the outer leaflet and glycerophospholipids (GPLs) at the inner leaflet contributes to the high intrinsic level of resistance to antibiotics. OM asymmetry is mainly maintained by the Mla system (MlaA-MlaBCDEF) [3]. MlaA, also known as VacJ (Virulence-associated chromosome locus J) was first identified in *Shigella flexneri* as a gene required for the intracellular spreading bacteria [4]. MlaA removes GPLs from the outer leaflet of OM and delivers it to the MlaFEDB complex at the inner membrane (IM) via the periplasmic substrate-binding protein MlaC [3]. Genetic and structural works suggest that the Mla pathway is retrograde (GPLs movement from OM to IM) [5]. However, based on early biochemical results that show ATP-independent transfer of

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GPLs from MlaD to MlaC [6,7], several groups have advocated that transport could happen in an anterograde fashion (from IM to OM) [8]. Additional biochemical studies, more relevant from bacterial physiology, demonstrated that ATP-dependent transport was predominantly retrograde [9], and the ATP hydrolysis is key to prevent the spontaneous transfer of PLs from MlaD to MlaC thus blocking anterograde transport [10]. Currently, functional, structural [9] and biochemical [10] studies strongly favour the retrograde transport as nicely reviewed in Ref. [11].

MlaA protein is required for the intracellular spreading of *S. flexneri* and is associated with bacterial transmission [4,12]. A putative role of MlaA in evading the complement system in *Actinobacillus pleuropneumoniae* MD12 *ΔmlaA* has also been demonstrated [13]. Among clinical isolates, non-typeable *Haemophilus influenzae* adapts to inflammation in the lower respiratory tract through increased expression of *mla* genes to minimize recognition by bactericidal anti-oligosaccharide antibodies [14]. Similar to *Glaesserella parasuis*, loss of serum resistance in *mlaA* mutants correlated with increased binding of natural immunoglobulin M in serum as well as anti-oligosaccharide monoclonal antibodies [15]. At the molecular level, MlaA is known to interact with other OM proteins. In *Escherichia coli*, MlaA interacts with OmpC, a trimeric porin that typically allows the passage of hydrophilic solutes across the OM [16–18]. In *Klebsiella pneumoniae*, MlaA is co-purified with OmpF. *P. aeruginosa* and *Acinetobacter baumannii*, both lack OmpC and OmpF porins despite they express MlaA homologs [11]. Other proteins like OprO [19] and OprP [2] in *P. aeruginosa* and DcaP [20] in *A. baumannii*, could play a role in MlaA activity by stabilizing the structure and orientation of MlaA in the OM [18].

P. aeruginosa poses threat by its ability for rapid adaptation to various growth modes and environmental conditions, controlled in part through interwoven quorum-sensing (QS) circuits; *las*, *rhl*, and *pqs* which regulate the global expression of numerous virulence-associated genes [2,21]. The virulence factors, whether secreted (e.g., proteases, rhamnolipids, pyocyanin), or cell-associated like pili, flagella, and LPS, play a critical role in the cell motility and biofilm formation [22].

Bacterial pathogenicity is dependent upon the inflammatory response [23] induced by flagella, pili, and LPS. Lipid A part of LPS acts as pattern-associated molecular patterns (PAMPs). They are recognized by the Toll-like receptor 4 (TLR4) resulting in secretion of pro-inflammatory cytokines, required for pathogen's eradication. LPS varies structurally between different species of Gram-negative bacteria and certain lipid A forms provide a benefit in the binding TLRs and in the secretion of pro-inflammatory cytokines [24]. Interestingly, modulation of inflammatory response and *P. aeruginosa* clearance is also mediated by rhamnolipids [25].

Mla system in *P. aeruginosa* is encoded by PA2800 (MlaA or VacJ) and PA4456-PA4452 (MlaBCDEF) [26]. In addition, PA3238 and PA3239 (renamed MlaY and MlaZ, respectively) are also found to be involved in maintaining OM lipid asymmetry in *P. aeruginosa* [27]. In this study, we explored the role MlaA, an OM lipoprotein in bacterial physiology with many important links to bacterial virulence. It allows us to better understand how *P. aeruginosa mlaA* knockout impacts antibiotic susceptibility, bacterial virulence and innate immune response, as well as the pathways involved.

By using *P. aeruginosa* ATCC27853 (WT) and its isogenic *ΔmlaA* strain, we compared the antibiotic susceptibility, motility, virulence factor secretion and biofilm formation as well as pro-inflammatory cytokines secretion. Based on the critical role of LPS on inflammatory response, we also determined the effect of two antibacterial compounds interacting with LPS; colistin and 3′,6-dinonyl neamine (3′,6-diNn) in both *P. aeruginosa* WT and *ΔmlaA*. Colistin functions by displacing cations between LPS molecules and leading to membrane disruption [28]. 3′,6-diNn is an amphiphilic aminoglycoside derivative which binds to LPS [29], and impairs the effect of MreB, a cardiolipin dependent cytoskeleton protein [30].

This study contributes to the understanding of effect of OM asymmetry and more specifically of the deleted *mla* component (*mlaA*) on antibiotic susceptibility, secretion of virulence factors, motility, biofilm formation, and cytokines production in the search of new strategies to fight *P. aeruginosa* infections.

2. Materials and methods

2.1. Chemicals

LPS from *P. aeruginosa* serotype 10 was supplied from Sigma–Aldrich (St. Louis, MO). 3′,6-dinonyl neamine (3′,6-diNn) was synthesized by Decout and colleagues (University Grenoble Alpes, France). BODIPY-TR-cadaverine (BC) was obtained from Molecular Probes (Invitrogen, Carlsbad, CA, United States). Cation-adjusted Muller Hinton broth (CA-MHB), from Sigma–Aldrich (St. Louis, MO, United States); cell culture media, from Gibco/Life Technologies Corporation (Paisley, United Kingdom); Mueller Hinton broth (MHB) and tryptic soy agar (TSA), from VWR (Radnor, PA, United States); and primers, from Eurogentec (Liège, Belgium). Other reagents or antibiotics were purchased from E. Merck AG (Darmstadt, Germany), Sigma–Aldrich–Fluka (Lyon, France), or Acros Organics (Illkirch, France).

2.2. Bacterial strains

P. aeruginosa WT (ATCC27853) was provided by Prof. R. Vanhoof from the Pasteur institute (Brussels, Belgium). *P. aeruginosa ΔmlaA* (PA2800) mutant was created using two-step allelic exchange [31]. The sequence information for the gene was obtained from Pseudomonas Genome Database (http://www.pseudomonas.com/). The 700 bp flanking region of *mlaA* was amplified by PCR (TransTaq High Fidelity, TransGen Biotech) using a forward and a reverse primer with the overlapping regions of the pEXG2 suicide plasmid using the 4 primers given in Table 1. The pEXG2plasmid was amplified and linearized by PCR (TransTaq High Fidelity, TransGen Biotech) using primers in Table 1. The thermo cycles were programmed as follows: initial denaturation at 95 °C for 15 s, followed by 30 cycles of 60 °C for 15 s, annealing temperature at 72 °C for 30 s and a final extension at 72 °C for 5 min. The PCR products were analyzed on agarose gel by electrophoresis. The fragments were assembled using Gibson assembly (New England Biolabs, Evry, France) at 50 °C for 1 h. The assembled plasmid was cloned into competent *E. coli* DH5α strain by heat shock and the positive clones were selected on gentamicin (10 mg/l) LB agar plates. The clones were verified by Sanger sequencing to check the presence of the *mlaA* inserts into pexG2 plasmid. Then, pEXG2-*ΔmlaA* was extracted with Nucleospin RNA Mini (Macherey-Nagel, Hoerdt, France) and transformed into *E. coli* strain Sm10 (λpir) by heat shock. *P. aeruginosa* ATCC27853 and *E. coli* strain Sm10 were grown overnight in LB at 42 °C and at 37 °C with gentamicin (10 mg/l), respectively. Then, the plasmid was transferred into *P. aeruginosa* ATCC27853 by conjugation for first homologous recombination. After 4 h of coculture the *P. aeruginosa* ATCC27853 containing the plasmid were selected on irgasan (20 mg/l) and gentamicin (15 mg/l) plates at 37 °C for 24–48 h. Irgasan is used to eliminate *E. coli* Sm10 (λpir) and gentamicin for the plasmid selection. The colonies were then inoculated on sucrose (5%) no salt LB agar plates used as a counter selection to cure the plasmid and to force the second homologous recombination. The targeted gene (*mlaA*) from the clone that grew on sucrose was amplified by PCR (TransTaq High

Table 1
Primers used for creating *P. aeruginosa* ATCC27853 *ΔmlaA*, *P. aeruginosa* ATCC27853 *ΔmlaA att7::mlaA* and for performing qRT-PCR of inflammatory cytokines.

Function	Primer's sequence in direction; 5′−3′ (on PAO1 genome)
Gibson assembly of plasmid pEXG2 and flanking region of *mlaA* − upstream region	F-ACATTATACGAGCCGGAAGCATAAATGTAAAGCAAGCTTTGTCTTCCAAGTAGGCGGCG R-GGGTCGACTTAGAAGTCGTCCTCGCCGCGGGCGCGCATTGCTG
Gibson assembly of plasmid pEXG2 and flanking region of *mlaA* − downstream region	F-TCGACTTAGAAGTCGTCCTCGCCGCGGGCGCGCATT R-CCGCGTTCAGCGCGGTCGGGGATCCCCGGGCTCGAGCTCGAATTCGGTACCTTAATTAA
Linearizing plasmid	F-ATAGTGAACGGCAGGTAAGC R-GATCGTGCTCCTGTCGTTGA
To check plasmid insertion	F-ATAGTGAACGGCAGGTAAGC R-GATCGTGCTCCTGTCGTTGA
To check *vacJ* deletion	F-GATCTGCAGGCCCTGCAGAC R-TGCAGGCCGAAACGCTCG
Gibson assembly of pUC18-Tn7T-P*mlaA*-*mlaA* vector amplification	F-AGGTGGAGGACGACTTCTAATGCAGGCCAACCAGATAAGTG R-ACGCGCCGCGATCGCCTGACGATCGAATTAGCTTCAAAAGCGCTC
Gibson assembly of pUC18-Tn7T-P*mlaA*-*mlaA* fragment amplification	F-CTTTTGAAGCTAATTCGATCGTCAGGCGATCGCGGC R-ACTTATCTGGTTGGCCTGCATTAGAAGTCGTCCTCCACCTCG

Gene	NCBI reference	Primer's sequence (5′−3′)
tnf-α	NM_000594.4	F-AAAACAACCCTCAGACGCCA R-AGAGGCTGAGGAACAAGCAC
il-1β	NM_000576.3	F-CTTAAAGCCCGCCTGACAGA R-ACACTGCTACTTCTTGCCCC
il-6	NM_001371096.1	F-TGCAATAACCACCCCTGACC R-GCTACATTTGCCGAAGAGCC
il-8	NM_000584	F-TCTGCAGCTCTGTGTGAAGG R-CCAGTTTTCCTTGGGGTCCA
mcp-1	NM_002982	F-AGCAGCAAGTGTCCCAAAGA R-TCTGGGGAAAGCTAGGGGAA
iNOS	NM_000625	F-ACAGGAGGGGTTAAAGCTGC R-GAGGCTCCGATCAATCCAGG
gadph	NM_002046	F-GATTTGGTCGTATTGGGCGC R-TTCCCGTTCTCAGCCTTGAC

Fidelity, TransGen Biotech) using check primers (Table 1) and migration on agarose gel was done by electrophoresis to check the mutants that have been deleted for the *mlaA* gene.

2.3. *Single-copy* mlaA *complementation*

Wild-type *mlaA* under control of its native promoter (P_{mlaA}) was cloned into the mini-Tn7 vector, which was then used to integrate a single copy of P_{mlaA}-*mlaA* at the *attTn7* site into the chromosome of *P. aeruginosa* ATCC27853 *ΔmlaA* by transposition as previously described [32]. Briefly, the wild-type allele of *mlaA* including its promoter P_{mlaA} was amplified by PCR from *P. aeruginosa* ATCC27853 WT and integrated into the mini-Tn7 vector by Gibson assembly. Nanopore sequencing was used for full-length plasmid verification and 2 positive clones were conserved. Recombinant mini-Tn7T-*mlaA* vector was co-transformed together with the pTNS2 helper plasmid into *P. aeruginosa* ATCC27853 *ΔmlaA*. The pTNS2 plasmid encodes the Tn7 site-specific transposition genes *tnsABCD*, required for transposition of P_{mlaA}-*mlaA* at the *attTn7* integration site of *P. aeruginosa*. Complementation candidates were selected on gentamycin 30 μg/ml and whole genome sequenced to verify the absence of unwanted mutations in the chromosome. Specific details of the subcloning strategy used for each construct are available upon request.

2.4. *Growth characteristics of* P. aeruginosa *WT (ATCC27853) and* ΔmlaA

P. aeruginosa WT (ATCC27853) and *ΔmlaA* strains were grown on TSA plate overnight at 37 °C. A colony was picked and grown in Luria−Bertani (LB) broth at 37 °C with 130 rpm agitation, overnight. Next day, the bacterial suspension was diluted to obtain OD_{620nm}: 0.05 and incubated at 37 °C with 130 rpm agitation. The OD_{620nm} was measured at each hour for 8 h and then at 24 h. For antibiotic treatment, both *P. aeruginosa* strains were grown until mid-exponential phase (OD_{620nm}: 0.6−0.8) in LB and treated for 1 h with colistin or 3′,6-diNn.

2.5. *Intracellular survival*

The human monocytic leukaemia cell line, THP-1 macrophages (clone ATCCTIB-202; American Type Culture Collection) were incubated with *P. aeruginosa* WT and *ΔmlaA* with multiplicity of infection (MOI) of 10 for 180 min. After 1 h, extracellular bacteria were killed with gentamicin (50 mg/l). Then, at each 30 min, cells were washed with 1× PBS, lysed, and plated on LB agar plates. Colony Forming Units (CFUs) were counted after plating and overnight incubation of serially diluted aliquots.

2.6. *Scanning electron microscopy (SEM) for morphology*

To observe the morphological changes, CM12 Philips electron microscope at 80 kV with the secondary electron detector was used. *P. aeruginosa* WT (ATCC27853) and the *ΔmlaA* strain at OD_{620nm}: 0.8 [33] were washed with 1× PBS twice and immobilized on IBIDI chambers with poly-L-Lysine (0.1 mg/ml). After 10 min of incubation at room temperature, they were washed with 1× PBS to remove the excess bacteria from the coverslips. Fixator (phosphate buffer + 1% glutaraldehyde) was added to each well and then post-fixed with 1% osmium tetroxide in cacodylate buffer for 2 h at 4 °C. Samples were washed to remove excess osmium tetroxide and dehydrated with graded series of ethanol and coated with 10 nm of gold. The experiment was done in triplicates, 10 images per condition were taken each time and 100 bacterial cells were considered for each condition.

2.7. Antibiotic susceptibility assay

Minimum inhibitory concentrations (MICs) of antibiotics were assessed by microdilution in cation adjusted-Mueller Hinton Broth according to Clinical and Laboratory Standard Institute (2018).

2.8. Macrophage differentiation and LPS stimulation

THP-1 cells were grown in RPMI media supplemented with 5% foetal bovine serum (FBS, Gibco) at 37 °C in 5% CO_2. THP-1 monocytes were induced to differentiate into macrophages in a 12-well plate by treating cells with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich) for 48 h. To induce inflammatory response, THP-1 macrophages were incubated at 37 °C for 2 h with either *P. aeruginosa* WT (ATCC27853) and *ΔmlaA* with 10^6 cells/ml at multiplicity of infection (MOI) of 10. Non-induced cells were considered as negative control (vehicle). For THP-1 stimulation with LPS, LPS extraction was performed by using the intron biotechnology kit and quantified using Purpald assay. THP-1 macrophages were stimulated with different concentrations (0, 50, 100, and 1000 ng per 10^6 cells) of extracted LPS with *P. aeruginosa* WT and *ΔmlaA* before and after treatment with 3′,6-diNn and colistin for 2 h. Then, cells were detached using trypsin for 3 min and collected in a 1.5 ml microtube. The cells were centrifuged to separate supernatant for the enzyme-linked immunosorbent assay and the pellet for analyzing the mRNA expression.

2.9. Total RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from THP-1 cells using TRIzol method. Briefly to 10^6 THP-1 macrophages, 400 μl of TriPure™ isolation reagent was added, passed through pipette to homogenize, and incubated at room temperature for 5 min. Then, 400 μl of chloroform was added, and the mixture was vortexed and centrifuged at 12,000 *g*, 10 min. The upper colourless phase (±200 μl) containing RNA was transferred into a new tube. 400 μl of isopropanol was added and the solution was mixed, incubated at room temperature for 5 min, and centrifuged at 12,000 *g*, for 10 min. The supernatant was discarded. To the pellet, 400 μl of ethanol (75%) was added and the mix was vortexed before centrifugation at 12,000 *g*, 10 min. RNA as a pellet was recovered and 15 μl of RNase-free water was added.

One microgram of total RNA was heated at 70 °C for 5 min, cooled down at 4 °C and then mixed with random primers and reverse transcriptase to synthesize complementary DNA (cDNA). Reverse transcription was performed on iCycleriQ (BIO-RAD, USA) with thermal cycler programming as follows: 25 °C for 5 min; 42 °C for 60 min; 70 °C for 15 min, 4 °C. Real-time PCR was performed with SYBR green (SsoAdvanced™ Universal SYBR® Green Supermix, BIO-RAD, USA) in a CF96™ Real-Time System (C1000 Touch™ Thermal cycler, BIO-RAD). GADPH was used as a house-keeping gene to normalize the expression of the genes in the respective strains.

2.10. Cytokines quantification by enzyme-linked immunosorbent assay (ELISA)

Tumour necrosis factor-α (TNF-α) and Interleukin-1β (IL-1-β) were quantified in the supernatant collected as described above with human ELISA kits (DuoSet; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.11. Bodipy™-TR-cadaverine (BC) displacement assay

Binding to the lipid A region of LPS was determined using the BC displacement assay [34], in which the probe bound to cell-free LPS is self-quenched but fluoresces when released in solution. Colistin and 3′,6-diNn were used at increasing concentrations (0–10 μM). The experiment was performed as described in Ref. [29] with the excitation and emission wavelengths at 580 nm and 620 nm, respectively.

2.12. Quantification of Pseudomonas Quinolone Signal (PQS) and virulence factors

2.12.1. PQS extraction and quantification

PQS was extracted from 20 ml of culture supernatant of *P. aeruginosa* using 20 ml acidified ethyl acetate (0.01% acetic acid) [35]. The organic phase was moved to a different tube, and the solvent was evaporated using a nitrogen stream. PQS was dissolved in methanol and spotted on HPTLC. A 60 F_{254} normal phase silica HPTLC plate was soaked in 5% KH_2PO_4 and activated at 100 °C for 1 h. The plate was placed in the HPTLC chamber pre-saturated with the mobile phase (2-Propanol/Ethyl acetate (4:6)). PQS (2-Heptyl-3-hydroxy-4-quinolone) was utilized as a positive control and to quantify PQS produced by *P. aeruginosa*. The densitometry of the applied PQS spots was measured using a CAMAG TLC scanner at 254 nm [36].

Elastase activity was determined by Elastin Congo Red assay [22]. Briefly, bacteria were grown until mid-exponential phase (OD_{620nm}: 0.6–0.8) in LB at 37 °C with 130 rpm agitation. Bacteria were centrifuged (20,000 *g*, 5 min) and pellet was resuspended in water the OD_{620nm}. After passing through filters (0.22 μm), 50 μl of supernatants was added to 1 ml of Tris-maleate buffer (0.1 M Tris, 1 mM $CaCl_2$ pH 7.2) containing 20 mg of Elastin Congo Red. The suspension was incubated for 18 h at 37 °C under agitation (130 rpm). Later, 0.1 ml of 0.12 M EDTA was added and placed on ice. The insoluble Elastin Congo Red was removed by centrifugation (5000 rpm, 2 min) and OD_{495nm} was measured. Elastase activity was estimated by dividing OD_{495nm} of the filtered supernatant by OD_{620nm} of the resuspended pellet.

2.12.2. Pyocyanin assay

P. aeruginosa WT (ATCC27853) and *ΔmlaA* were grown overnight. Bacterial supernatant was recovered by centrifugation. From 5 ml of supernatant, pyocyanin was extracted with 3 ml of chloroform. From the chloroform phase, pyocyanin was re-extracted into 1 ml 0.2 N HCl. The absorbance was measured at 520 nm [37].

2.12.3. Rhamnolipids

P. aeruginosa WT (ATCC27853) and *ΔmlaA* were grown until mid-exponential phase (OD_{620nm}: 0.6–0.8) in LB at 37 °C with 130 rpm agitation. The bacterial cells were pelleted by centrifugation and 2 ml of supernatant was used to analyze rhamnolipids by orcinol-sulphuric acid assay [38]. Rhamnolipids were extracted twice with 600 μl diethyl ether. The pooled ether extracts were evaporated and 100 μl water, 100 μl 1.6% orcinol, 800 μl sulphuric acid were added. The suspension was heated to 80 °C, agitating at 175 rpm and the absorbance was measured at 421 nm. Standard curve was prepared by different concentrations of rhamnose, 0–50 μg/ml).

2.13. Bacterial motility was determined by swimming, swarming, and twitching

P. aeruginosa WT (ATCC27853) and *ΔmlaA* strains were grown in LB overnight and were inoculated either on swimming plates prepared with tryptone (0.1%, wt./vol), yeast extract (0.05%, wt./vol), NaCl (0.5%, wt./vol) and agar (0.3% wt./vol), or on twitching plates containing tryptone (0.1%, wt./vol), yeast extract (0.5%, wt./vol), NaCl (0.5%, wt./vol) and agar (1% wt./vol). For swarming, bacteria

were inoculated on plates consisted of nutrient broth (0.8%, wt./vol), glucose (0.5%, wt./vol) and agar (0.5%, wt./vol). The plates were incubated at 37 °C for overnight. The distance travelled by bacteria was measured in mm with a measuring scale [39].

2.14. Biofilm biomass and thickness

2.14.1. Biofilm biomass

P. aeruginosa WT and *ΔmlaA* strains were grown in 200 μl of LB media for 24 h in a 96 well plate. The biofilm was washed with 200 μl of 3-morpholinopropane-1-sulfonic acid (MOPS) to remove the planktonic bacteria and dried at 60 °C for 1 h. Then, 0.5% crystal violet was added to the wells and incubated for 15 min, and excess crystal violet was removed with running water. Biofilm was solubilized in 66% acetic acid and incubated for 1 h in a dark place. The biomass was calculated from the absorbance values measured at 570 nm using a spectramax M3 plate reader.

2.14.2. Biofilm imaging

The bacterial suspensions were prepared in 24-well polystyrene plates with a coverslip at the bottom. After 24 h, the planktonic bacteria were taken out, and the biofilms were washed with PBS and stained with SYTO 9. After 15 min, the dye was removed, and the coverslips were washed with PBS. The coverslips were mounted on a Carl Zeiss cell observation spinning disk microscope with a 40× objective. SYTO 9 was visualized in the green channel (Excitation/Emission: 488/502−538 nm), and images were taken with a resolution of 1388 × 1040 pixels using the Z-stacks scanning mode. ZEN 2.6 (blue edition) software was used to make the 3D images of the biofilms and measure their thicknesses.

3. Results

3.1. Deletion of mlaA in P. aeruginosa ATCC27853 did not impact growth, survival in THP-1 and bacterial morphology

To examine the effect of *mlaA* deletion on growth rate and survival during culture conditions, we monitored the OD_{620nm} in LB media and the CFU/ml in THP-1 macrophages respectively over time. *ΔmlaA* did not impact growth as compared to the isogenic WT strain (Fig. 1A). Complemented strains (*ΔmlaA att7::mlaA*) also showed similar growth rate as compared to WT or *ΔmlaA* strains (Fig. 1A). No significant difference of intracellular survival of WT and *ΔmlaA* strains was observed over 180 min when THP-1 macrophages were infected with WT and *ΔmlaA* strains (Fig. 1B). Examination of cell morphology by scanning electron microscopy showed *mlaA* deletion did not induce major alterations to the cell morphology (Fig. 1C).

3.2. Deletion of mlaA increased susceptibility of P. aeruginosa ATCC27853 to fluoroquinolones and polymyxin E

To investigate the potential effect of *mlaA* deletion on antibiotic susceptibility, we determined the MIC values to fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin), aminoglycosides (amikacin, gentamicin, tobramycin, neomycin and neamine), an amphiphilic aminoglycoside derivative (3′,6-diNn), a polymyxin (colistin) and carbapenems (imipenem and meropenem) (Table 2). Fluoroquinolones inhibit DNA gyrase, aminoglycosides bind to ribosomal RNA and inhibit protein synthesis whereas 3′,6-dinonyl neamine and colistin target bacterial membranes and carbapenems the cell wall.

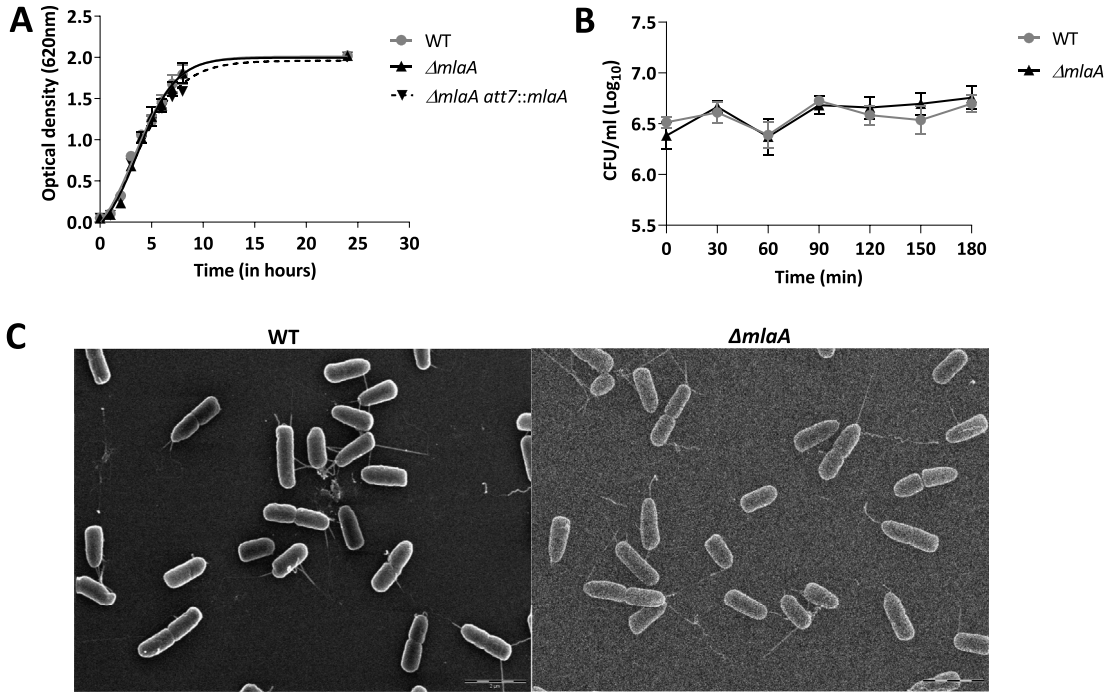

Fig. 1. *P. aeruginosa* ATCC27853 WT and *ΔmlaA*, growth curve, intracellular survival in THP-1 cells and cell morphology. (A). Growth of strains in LB media by measuring OD_{620nm} values over 8 h and at 24 h; (B). Infection rate of *P. aeruginosa* WT and *ΔmlaA* in THP-1 macrophages by determining colony forming units (CFUs) every 30 min for 3 h; (A, B). Data are mean ± SEM (triplicates from three independent experiments); (C). Scanning electron microscopy of *P. aeruginosa* WT and *ΔmlaA* grown until mid-exponential phase (Scale bar 2 μM, N = 3, n = 10 images per condition).

Table 2
Antibiotic susceptibility assay.

Class of antibiotics	Antibiotics	Target	MICs		MICs ratio
			WT (mg/l)	*ΔmlaA* (mg/l)	
Aminoglycosides	Amikacin	30s ribosomal subunit	1	1	–
	Gentamicin		1	1	–
	Tobramycin		0.25	0.25	–
	Neomycin		32	32	–
	Neamine		512	512	–
Amphiphilic aminoglycoside derivative	3′,6-dinonyl neamine	LPS & GPL domains	2	2	–
Polymyxin	Colistin	LPS	1	0.5	2
Fluoroquinolones	Ciprofloxacin	DNA gyrase	0.125	0.0625	2
	Levofloxacin		0.5	0.125	4
	Moxifloxacin		2	0.25	8
Carbapenems	Imipenem	Bacterial cell wall	4	4	–
	Meropenem		0.5	0.5	–

All MICs were determined in at least 2–3 independent experiments.

Deletion of *mlaA*, led to decrease in MIC by 2× for colistin. Changes in MICs were also observed with fluoroquinolones with MICs decrease (2, 4 and 8 × MIC for ciprofloxacin, levofloxacin, and moxifloxacin, respectively) for *P. aeruginosa ΔmlaA* as compared with WT. These findings suggest that the absence of *mlaA* in *P. aeruginosa* increased susceptibility was an antibiotic class-specific action.

3.3. *Deletion of* mlaA *in* P. aeruginosa *ATCC27853 increased concentration of* Pseudomonas *Quinolone Signal (PQS) autoinducer and reduced rhamnolipids secretion without any effect on elastase activity and pyocyanin production*

MlaA was reported to influence the secretion of virulence factors in numerous Gram-negative bacteria [40,41]. In *P. aeruginosa*, we were wondering to what extent *mlaA* deletion induced stress and activation of PQS (2-heptyl-3-hydroxy-4-quinolone). Thus, in *P. aeruginosa* WT and *ΔmlaA* strain, we monitored PQS contents as well as production of one of virulence factor induced, pyocyanin. As PQS provides a link between *las* and *rhl* systems, being activated by *las* and repressed by *rhl* [42], we also determined the secretion of elastase and rhamnolipids, as the results from LasR and RhlR activation, respectively. After normalisation with the OD_{620nm}, the amount of PQS autoinducer for *P. aeruginosa ΔmlaA* was around 1.75 times higher that found in WT (Fig. 2A). Complemented strains (*ΔmlaA att7::mlaA*) showed PQS levels like those obtained for WT strains (Fig. 2A). Rhamnolipids secretion was reduced to 55.6% in *P. aeruginosa ΔmlaA* (30.0 ± 6.0 μg/ml) as compared to the WT (60 ± 13 μg/ml) (Fig. 2B). Regarding the elastase activity and pyocyanin production, no significant differences were observed (Fig. 2C and D) by comparing *P. aeruginosa* WT and *ΔmlaA*.

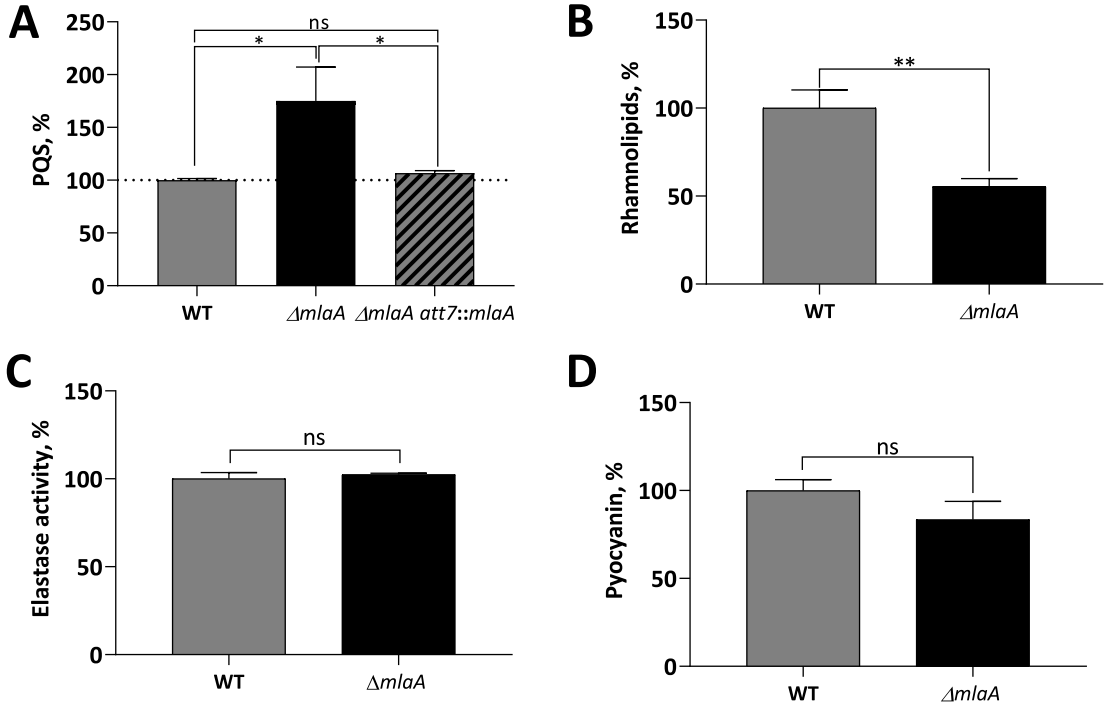

Fig. 2. Effect of *mlaA* deletion in *P. aeruginosa* ATCC27853 on PQS and secretion of virulence factors. (A). PQS was quantified by HPTLC and CAMAG TLC scanner at 254 nm; (B). Rhamnolipids were determined by orcinol-sulphuric acid assay; (C). Elastase activity was estimated by using Elastin Congo Red assay; (D). Pyocyanin was monitored by OD at 520 nm. All data are mean ± SEM (triplicates from three independent experiments). Results are expressed in relative percentage over WT $^{**}p < 0.01$, $^{*}p < 0.05$, $^{ns}p > 0.05$, One-way ANOVA with Tukey's multiple comparison test (A), Student's t-test (B, C, and D).

3.4. Deletion of mlaA impaired motility in P. aeruginosa ATCC27853

Since self-produced biosurfactant like rhamnolipids are required for swarming motility [43,44], we thus hypothesized a decreased in *P. aeruginosa* swarming induced by *mlaA* deletion. In addition to swarming, we also assessed the swimming, and twitching for *P. aeruginosa* WT and *ΔmlaA* strains. *P. aeruginosa ΔmlaA* displayed a decrease of swarming by 25% (11 ± 1 *versus* 15 ± 1 mm) but also a reduced twitching by 14% (17 ± 1 *versus* 20 ± 1 mm), and swimming by 22% (33 ± 2 *versus* 42 ± 2 mm) as compared *P. aeruginosa* WT, suggesting that *mlaA* might play a role in motility (Fig. 3). Swarming, swimming and twitching were restored in complemented strains (*ΔmlaA att7*::*mlaA*) to reach values similar to those found for WT strains (Fig. 3). Interestingly, round *P. aeruginosa* swarms, without branches were observed.

3.5. Deletion of mlaA decreased biofilm formation in P. aeruginosa ATCC27853

Decrease in motility can be related to mutation in flagellum affecting its attachment to the bacterial cell [45]. Moreover, flagellar and type IV pili are required for primary surface adhesion, cellular aggregation and biofilm formation in *Pseudomonas fluorescens* [46], *E. coli* [47] and *P. aeruginosa* PAO1 [48]. We thus hypothesized a decreased in biofilm formation in *P. aeruginosa ΔmlaA*. By fluorescence imaging with SYTO 9 and by colorimetric assay with crystal violet assay, we showed *P. aeruginosa ΔmlaA* produced a much thinner biofilm (25 ± 7 μm *versus* 130 ± 14 μm) with a lower amount of biofilm biomass as compared to the WT in LB media after 24 h (Fig. 4A and B). The deletion of *mlaA* did not affect the close relationship between motility and biofilm formation.

3.6. P. aeruginosa ΔmlaA (whole bacteria) increased TNF-α production by human macrophages as compared to WT strain

As bacterial virulence and host responses are closely linked, and as targeting Mla pathway can sensitize *P. aeruginosa* killing by the host immune response [49], we assessed this relationship in *P. aeruginosa* ATCC27853 *ΔmlaA*. We thus infected THP-1 macrophages with *P. aeruginosa* WT and *ΔmlaA* whole bacteria for 2 h of stimulation with multiplicity of infection (MOI) of 10 and determined cytokine production including inducible nitric oxide synthase (iNOS) by qRT-PCR at mRNA level and by ELISA at protein level (Fig. 5). *P. aeruginosa ΔmlaA* induced highest change in the mRNA expression of pro-inflammatory cytokine *TNF-α* (3-fold) followed by *iNOS* (1.6-fold) over WT. No change in the mRNA expression of *IL-1β*, *IL-6*, *IL-8*, and *MCP-1* was observed (Fig. 5A). At the protein levels (Fig. 5B and C), for *P. aeruginosa* WT and *ΔmlaA*, we observed a higher release of IL-1β (1310 pg/ml) and TNF-α (1455 pg/ml) respectively, in the THP-1 macrophages as compared to the non-induced THP-1 (vehicle; 27 pg/ml [Fig. 5B] and 40 pg/ml [Fig. 5C]). IL-6 protein levels were also checked in the samples after 2 h of infection but could not be detected (data not shown). When compared, *P. aeruginosa ΔmlaA* infected cells produced increased amounts in TNF-α (Fig. 5B) and a slight decrease but non-significant in IL-1β than WT (Fig. 5C).

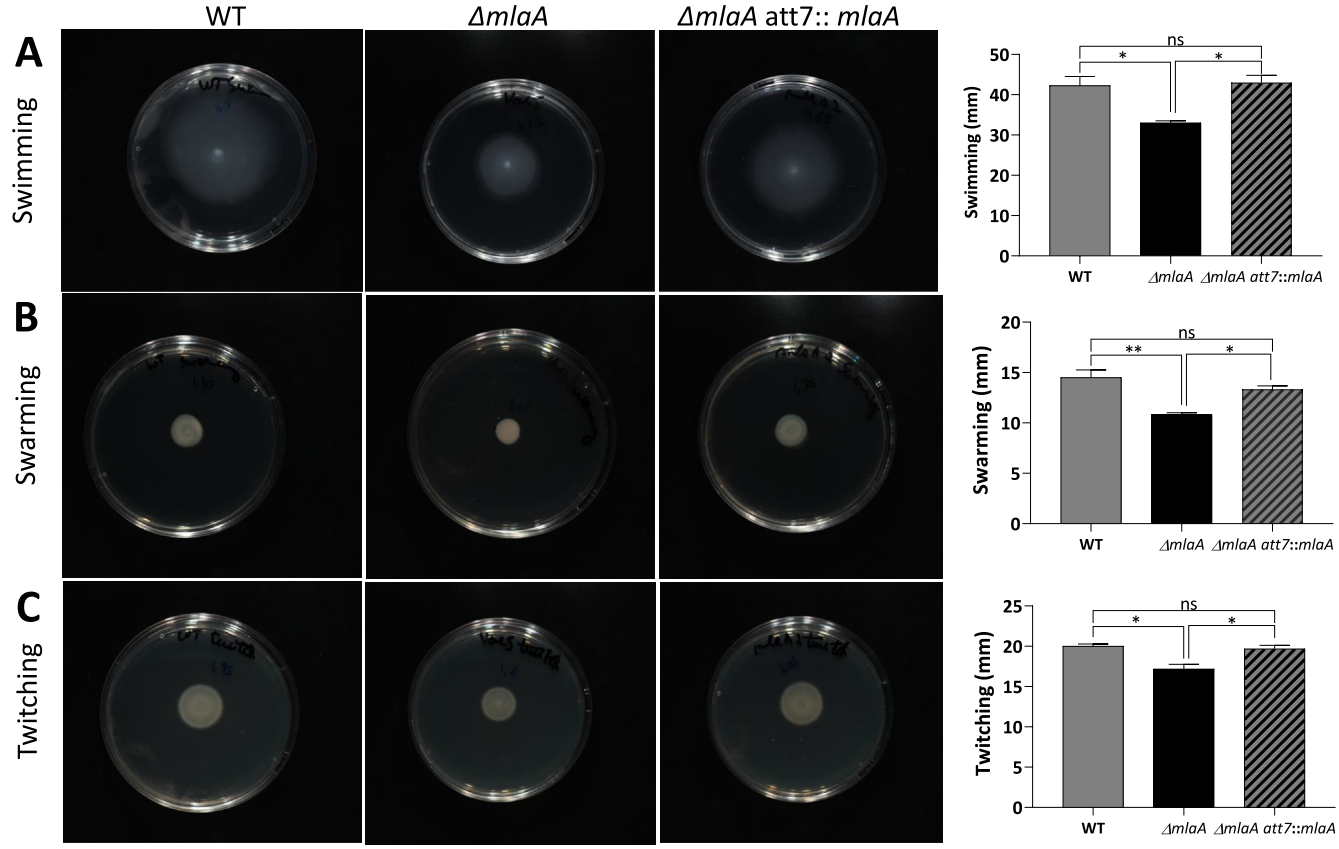

Fig. 3. Effect of *mlaA* deletion on motility in *P. aeruginosa* ATCC27853. (A). Swimming; (B). Swarming; (C). Twitching. All experiments were repeated on 3–5 plates and data is represented as mean ± SEM. **p < 0.01, *p < 0.05, ^{ns}p > 0.05 One-way ANOVA with Tukey's multiple comparison test.

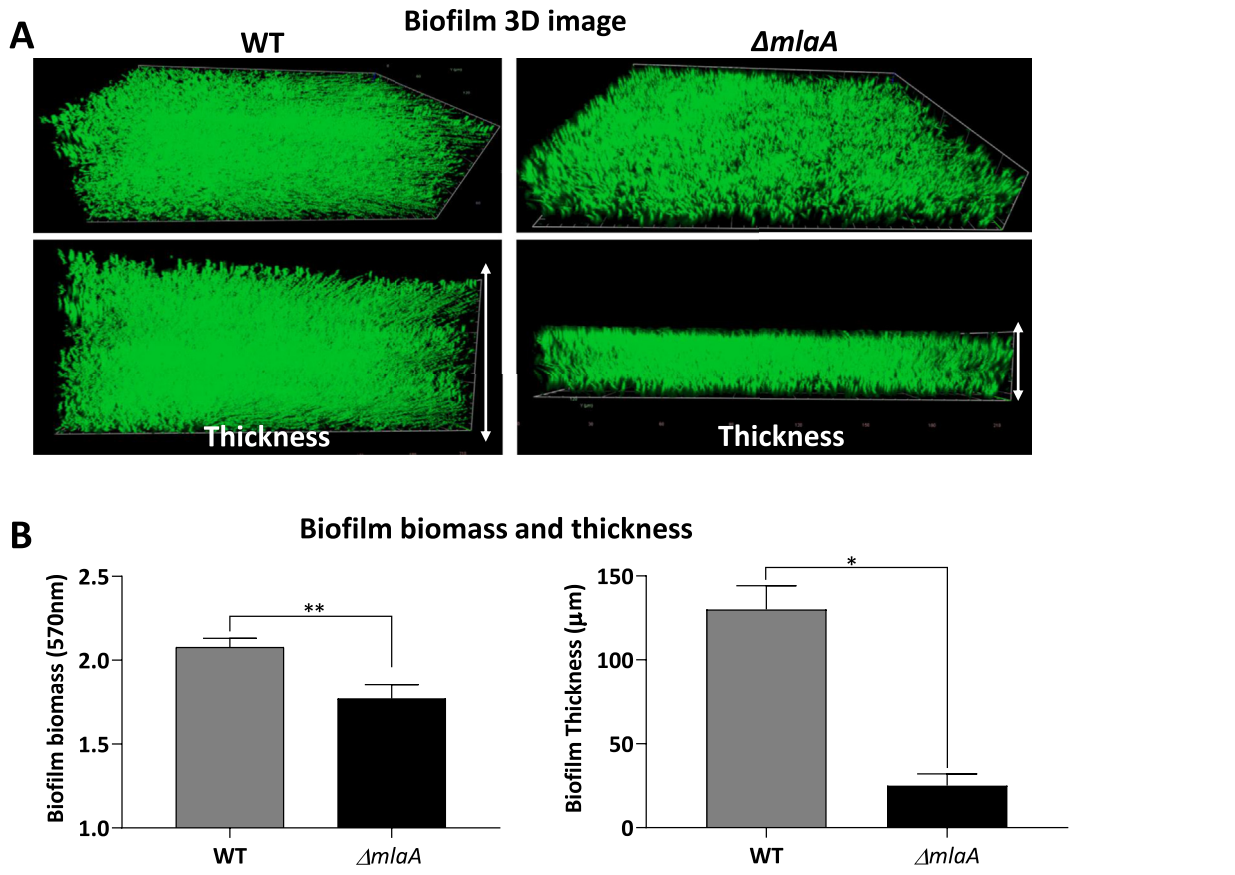

Fig. 4. Effect of *mlaA* deletion on thickness and biomass of biofilm of *P. aeruginosa* ATCC27853. The biofilm was formed in LB media after 24 h. The thickness was calculated from fluorescence imaging (SYTO 9) and biomass was obtained by measuring OD_{570nm} (crystal violet assay). Results are expressed in relative percentage over WT. Data are mean ± SEM (triplicates from three independent experiments). **p < 0.01, Student's t-test.

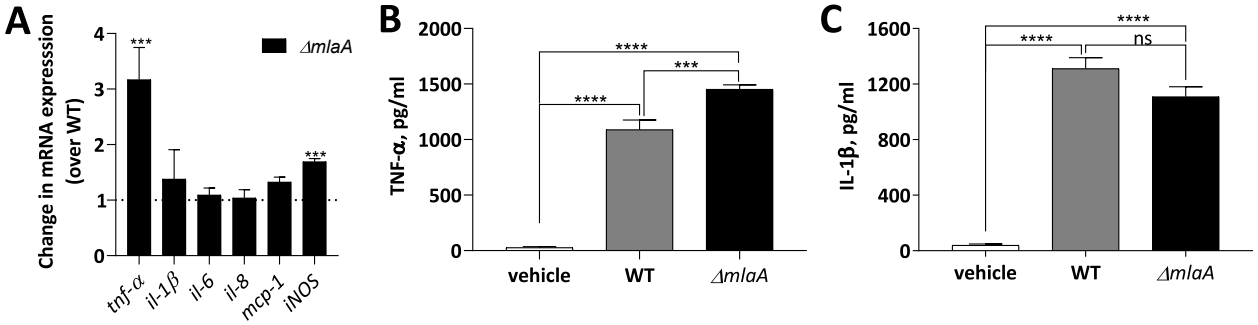

Fig. 5. Transcription and protein profile of inflammation related cytokines by THP-1 in response to *P. aeruginosa* ATCC27853 WT and *ΔmlaA* whole bacteria. The 10^6 THP-1 cells were either infected with WT or the *mlaA* deficient strain (*ΔmlaA*) and incubated for 2 h, MOI 10. (A). mRNA expression of cytokines; tumour necrosis factor-α (*TNF-α*), interleukin-1β (*IL-1β*), interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), Monocyte Chemoattractant Protein-1 (*MCP-1*) and inducible nitric oxide synthase (*iNOS*); (B). TNF-α; (C) IL-β production in the culture supernatant by *P. aeruginosa* WT and *ΔmlaA*. All experiments were repeated three times and data is represented as mean ± SEM. ****p < 0.0001, ***p < 0.001, *p < 0.05, ^{ns}p > 0.05, One-way ANOVA.

3.7. LPS extracted from P. aeruginosa ΔmlaA *induced dose-dependent increase in TNF-α by THP-1 human macrophages*

In a preliminary experiment, we monitored the Bodipy-TR-cadaverine displacement with colistin and 3′,6-diNn at varying concentrations ranging from 0 to 10 μM in *P. aeruginosa* WT and *ΔmlaA* (Fig. 6A). We hypothesized *mlaA* deletion alters LPS amounts and/or lipid A structures which in turn might alter the ability of colistin or 3′,6-diNn to bind to LPS and to displace Bodipy-TR-cadaverine from its binding to LPS. The de-quenching of LPS-bound Bodipy-TR-cadaverine fluorescence, manifests in emission intensity enhancements. Both colistin and 3′,6-diNn increased fluorescence intensity of Bodipy-TR-cadaverine after getting displacement from its binding to LPS. Interestingly, there was no difference in the ability of colistin to displace probe-bound to LPS from *P. aeruginosa* WT and *ΔmlaA* whereas *mlaA* deletion increased slightly the capacity of 3′,6-diNn for getting Bodipy-TR-cadaverine displacement (Fig. 6A).

To further investigate the association between the LPS from *P. aeruginosa* WT and *ΔmlaA*, with the cytokines production (TNF-α)

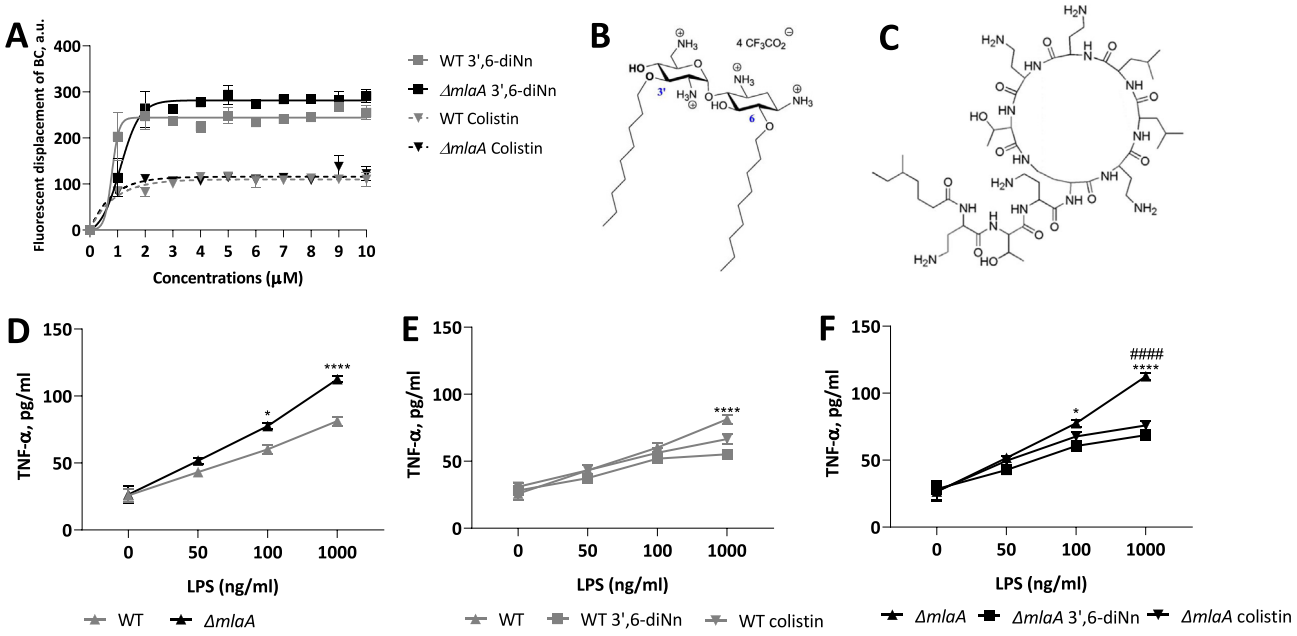

Fig. 6. (A). Displacement of Bodipy-TR-cadaverine bound to LPS from *P. aeruginosa* ATCC27853 upon the addition of 3′,6-diNn and colistin at increasing concentrations (0–10 μM); (B–C). Chemical structure of 3′,6-diNn, and colistin. (D–F). TNF-α released by THP-1 cells exposed to LPS extracted from *P. aeruginosa* ATCC27853 WT and *ΔmlaA* (D) before and after the treatment with 3′,6-diNn and colistin at 37 °C for 2 h at their MIC (E–F). All experiments were repeated three times and data is represented as mean ± SEM. * represents significance in comparison between 3′,6-diNn treated to WT or *ΔmlaA* and # represents significance between colistin treated to WT or *ΔmlaA* in each of LPS concentrations used, ****/#### $p < 0.0001$, ***/### $p < 0.001$, **/## $p < 0.01$, */# $p < 0.05$, Two-way ANOVA.

by THP-1 macrophages, dose-dependent (0, 50, 100, and 1000 ng/ml) response induced by extracted LPS from both strains was determined (Fig. 6D). We also investigated the potential effect of two membrane-active antibiotics such as 3′,6-diNn (Fig. 6B) and colistin (Fig. 6C) that interact with LPS [28,29], on the proinflammatory effect of LPS isolated from *P. aeruginosa* WT and *ΔmlaA* (Fig. 6E and F). TNF-α secreted by the THP-1 macrophages was determined after stimulation with LPS isolated before and after treatment with 3′,6-diNn and colistin in both strains.

As compared to control (vehicle), LPS extracted from both strains induced an increase in the release of TNF-α from THP-1 macrophages (Fig. 6D). The response was dose dependent with a higher response after challenge with LPS extracted from *P. aeruginosa ΔmlaA* at 100 and 1000 ng/ml (Table S1). The increase in TNF-α was significant from 100 ng/ml and 50 ng/ml for LPS extracted from *P. aeruginosa* WT and *ΔmlaA*, respectively (Table S1). Regarding the effect of colistin and 3′,6-diNn (Fig. 6E and F), they decreased the amount of TNF-α released with a significant effect observed at the highest dose (1000 ng/ml) (Table S1).

4. Discussion

P. aeruginosa cell envelope has a complex architecture. It includes an IM and an additional asymmetric lipid bilayer, the OM. The OM acts as selective barrier with low OM permeability, resulting in the high intrinsic resistance of *P. aeruginosa* [50]. The Mla system is involved in maintaining OM lipid asymmetry and is composed of six proteins that are distributed across the cell envelope, with MlaA located within the OM and in charge to remove excess of GPLs from the outer leaflet of OM. *P. aeruginosa* pathogenicity is also closely regulated by virulence factor production and biofilm formation [51] through quorum sensing systems [52]. This study investigated the impact of *mlaA* deletion on the antibiotic susceptibility, the secretion of virulence factors, biofilm formation and the inflammatory response as a whole.

The work was performed on *P. aeruginosa* ATCC27853 and its isogenic strain deleted in *mlaA*. The WT strain was initially isolated from a blood specimen in the Peter Bent Brigham Hospital in 1971 (Boston, USA), and widely used to survey antibiotics susceptibilities [53]. Seven prophages are present in its genome and ATCC27853 is characterized by several unique physiological features implying the striking ability of the strain to adapt to a variety of environmental niches and stress factors including a strong capability of the strain to form biofilms. Comparing *P. aeruginosa ΔmlaA* with its isogenic WT strain, we did not observe any significant impact on growth, in agreement with data reported for *Neisseria gonorrhoeae* [40], *E. coli* [54], *H. influenzae* [55] and *P. aeruginosa* PAO1 [49] or on cell morphology in accordance with data from *E. coli* [17].

The impact of *mlaA* deletion in *P. aeruginosa* ATCC27853 on antibiotic susceptibility was the first question raised in this work. In absence of antibiotic, no change in membrane permeability was observed between WT and *ΔmlaA*. In presence of antibiotics, *P. aeruginosa ΔmlaA* appeared to be more susceptible to fluoroquinolones and in a lesser extent to colistin, without any change in MIC observed for conventional aminoglycosides, an amphiphilic aminoglycoside derivative and carbapenems. The specific effect exerted by some classes of antibiotics was reported for other Gram-negative bacteria as well. The *mlaA*-knockout mutant of *E. coli* exhibited sensitivity to levofloxacin, chloramphenicol, oxacillin, cetyltrimethylammonium bromide (CTAB), and cholic acid, but was resistant to vancomycin [56], chlorhexidine [57]. In *N. gonorrhoeae*, knockout of *mlaA* sensitizes bacteria to defensins, polymyxin B, vancomycin, and ampicillin [40]. The mechanism by which the *mlaA* knockout mutant exhibits resistance or sensitivity is unclear but changes in OM surface, by accumulation of GPLs and changes in GPLs/LPS ratio, might have impact on the physico-chemical properties of the *P. aeruginosa* OM [58,59]. These are critical for the interactions between bacterial OM and amphiphilic antibiotics, which could in turn affect their diffusion through bacterial membranes, membrane integrity, absorption in OMVs, and/or activity of

efflux/influx proteins. These possibilities should be addressed in future studies.

Is the secretion of virulence factors modified with *mlaA* deletion in *P. aeruginosa* ATCC27853 was the second question asked. The deletion of *mlaA* increased PQS but decreased rhamnolipids secretion with no effect on elastase activity and pyocyanin secretion. This agrees with the closely interconnected pathways involved in the virulence response and increase of MVs induced by *mlaA* deletion might be in turn result in selective changes in the amounts of PQS and rhamnolipids. So, an increase of bacterial virulence was reported in *ΔmlaA* mutant strains of *E. coli* [56], *P. aeruginosa* PAO1 [41,56], *Vibrio cholerae* [60]. In contrast, a decrease in virulence was observed in *S. flexneri* [4,12], *A. baumannii* [61], *H. influenzae* [55] and *G. parasuis* [15], and *Burkholderia pseudomallei* [62]. Of note, changes in the secretion of virulence factors may also result from the alteration of the expression of LPS biosynthetic enzymes since the rhamnolipids biosynthetic pathway has common steps with LPS [63]. In addition, changes in the lipid A species through activation in the PmrAB-PhoPQ two-component system, which in turns induce lipid A palmitoylation, arabinosylation and hydroxylation might also have impact on the secretion of virulence factors [64].

In addition, compared to the WT strain, in *P. aeruginosa ΔmlaA* the biofilm biomass and thickness were reduced together with swimming, swarming, and twitching motility. This is consistent with data reported for *Xanthomonas citri* subsp. *citri* [65], *G. parasuis* [15], and *A. pleuropneumoniae* [13]. Hindering pili mediated twitching motility in a larger extent in *ΔmlaA* strain might impair micro-colony formation and colonization of surfaces. Rhamnolipids are required for *P. aeruginosa* swarming mobility [66], and the biofilm architecture [67,68] and thus could also play a critical role in virulence. Also, the activity of membrane proteins, including porins and/or efflux pumps could be regulated by their lipidic environment which, in turn, change in the entry/exit of virulence factors at variable extent.

The third question was about the effect of *mlaA* deletion on TNF-α release. We showed an increase in TNF-α released by THP-1 after incubation with *P. aeruginosa ΔmlaA* whole bacteria as compared to the WT. When THP-1 were incubated with LPS extracted from WT and *ΔmlaA* strains, a higher effect was observed when LPS was extracted from *P. aeruginosa ΔmlaA*. When bacteria were first treated with 3′,6-diNn or colistin, the extracted LPS induced a lower inflammatory response with a slightly higher effect induced by 3′,6-diNn as compared to colistin. LPS modifications could explain why alteration of the Mla system increased of TNF-α secretion by THP-1 cells. The lipid A isoforms have profound implications, owing to altered recognition by the TLR4 complex. Hexa-acylated lipid A form of LPS in *Pseudomonas* [69] is associated with a stronger TLR4 mediated inflammatory response while the lipid A with lower levels of acylation triggers reduced cellular responses. The dose-dependent increase of TNF-α release upon incubation with LPS, suggests the structural changes in the *P. aeruginosa ΔmlaA* LPS contribute to pro-inflammatory cytokines production in human cells. Interestingly, rhamnolipids also act as Pathogen-Associated Molecular Pattern molecules (PAMP) and their role should be investigated [70].

In conclusion, we demonstrated that deletion of *mlaA* in *P. aeruginosa* ATCC27853, (i) decreased fluoroquinolones MICs and to a lower extent of colistin, (ii) increased PQS but decreased rhamnolipids secretion along with biofilm biomass and thickness, and (iii) increased TNF-α release. Results from complementation experiments demonstrated these phenotypes are linked to *mlaA* and not to polar effect on adjacent genes or mutations elsewhere in the genome during construction process. At a whole, the observed phenotypes we demonstrated for *P. aeruginosa ΔmlaA* connect back to the known function of *mlaA*. As an example, since the first role of *mlaA* is devoted to intracellular spreading and virulence, deletion of *mlaA* is compatible with the decrease of biofilm thickness and reduced motility as we observed. The *in vivo* situation is probably much more complex, regarding the variability due to the strains, the bacterial population as the whole, and the site of infection. As an example of the importance of the bacterial *P. aeruginosa* strain, SNP (Single Nucleotide Polymorphism) distribution analysis in the genomes of PAO1 and ATCC27853 revealed that a large number of non-synonymous variant sites present in the two strains are concentrated in the regions and genes that encode surface associated proteins, such as those that encode flagellar components, pyoverdine receptor, transporters, and type 4 pili. Other factors like the multifaceted hierarchical QS systems in the clinical contexts [71] and the phenotypic heterogeneity of *P. aeruginosa* between patients [72] and/or the regional diversification of *P. aeruginosa* should be taken account. Thus, answering to the more general question to know if in *P. aeruginosa* inactivation of *mlaA* is an advantage *in vivo* is tricky. Investigating the molecular mechanisms of how the *mlaA* knockout upregulates bacterial virulence will help to elucidate the biological significance of the Mla system.

Declaration of competing interest

There is no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2023.104132.

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