Pseudomonas aeruginosa quorum sensing inhibition by clinical isolate Delftia tsuruhatensis 11304: involvement of N-octadecanoylhomoserine lactones

Milka Malešević1, Flaviana Di Lorenzo2, Brankica Filipić1,3, Nemanja Stanisavljević2, Katarina Novović2, Lidija Senerović1, Natalija Polović4, Antonio Molinaro2, Milan Kojić1 & Branko Jovčić1,5*

Pseudomonas aeruginosa is one of the most common opportunistic pathogens that use quorum sensing (QS) system to regulate virulence factors expression and biofilm development. Delftia sp. 11304 was selected among 663 Gram-negative clinical isolates based on its QS inhibitory activity against P. aeruginosa MMA83 clinical isolate. Whole genome sequencing identified this isolate as D. tsuruhatensis and revealed genetic armamentarium of virulence factors and antibiotic resistance determinants. Ethyl acetate extract of D. tsuruhatensis 11304 culture supernatant (QSI extract) prevented biofilm formation of P. aeruginosa MMA83, but was unable to cause biofilm decomposition. QSI extract showed a synergistic effect in combination with meropenem and gentamycin, against P. aeruginosa MMA83. A dose-dependent reduction of the virulence factors: elastase, rhamnolipid and pyocyanin production by P. aeruginosa MMA83 and significant downregulation of lasI, lasR, rhlI, rhlR, pqs and mvfR expression were observed. Matrix-assisted Laser Desorption Ionization (MALDI) mass spectrometry of D. tsuruhatensis 11304 QSI extract revealed the presence of N-acyl homoserine lactones (AHL) with chain lengths of C12 to C18. The main ion peak was identified as N-octadecanoylhomoserine lactone (c18-HSL). Commercial C18-HSL (20 µM) reduced pyocyanin production as well as mRNA level of the lasI gene. A novel AHL species, dihydroxy-N-octadecanoylhomoserine lactone, was also described.

Pseudomonas aeruginosa is one of the most significant opportunistic pathogens causing nosocomial infections with high morbidity and mortality, predominantly among immunocompromised and intensive care unit patients1–3. Pathogenic potential can be attributed to P. aeruginosa genomic plasticity and versatility which result in extensive genetic armamentarium of virulence factors and antibiotic resistance determinants5. Current increase in number of health care-associated infections caused by multidrug- (MDR) or extensively drug-resistant (XDR) P. aeruginosa isolates, as well as their worldwide spread has been considered as worrisome due to limited therapeutic options6. The lack of efficient therapeutic options for infections caused by MDR/XDR P. aeruginosa is driving research towards alternative therapeutic approaches such as targeting social behaviors involved in pathogenesis as well as bacteriophages and vaccines7,8.

P. aeruginosa cells communicate through quorum sensing (QS) system, i.e. by synthesizing small signal molecules, which depending on the density of the population correlate the regulation of virulence factors expression, biofilm development, production of secondary metabolites and interaction with hosts8–11. P. aeruginosa employs three major interconnected QS systems that function independently and dependently involving las, rhl, pqs pathways as well as novel candidate iqs pathway regulated by several QS signal molecules12. N-acyl homoserine lactones (AHLs) are the best characterized QS signal molecules. Different AHLs possess a homoserine lactone ring
an average GC content of 66.78%. The contig dataset was used to determine the functional analysis. Assembly of reads resulted in 199 contigs with the largest contig 337425 and an active compound31. Herein we analyzed inhibitory potential of anti-disease strategies targeting pathogens like P. aeruginosa QS signaling or interception of signal molecules is considered a key point for development of antibacterial and anti-disease strategies targeting pathogens like P. aeruginosa in medicine35. Indeed, interference with QS signaling by QSI-inhibitors (QSI) or interception of signal molecules by quorum quenching enzymes (QQE) results in a reduction of virulence regulated by QS. It is considered that QSI is a natural mechanism first developed either by QS-emitting organisms for the recycling or clearing of their own QS signals or by QSI organisms in the context of a competitive relationship with QS-signal-emitting organisms. Thus, bacteria that share ecological niche with P. aeruginosa during infections could be considered as promising producers of novel QSI molecules. Examples of the activity of QSI molecules, showing a successful reduction of siderophores, proteases, rhamnolipids secretion, as well as inhibition of biofilm formation have been previously documented. While those studies were mainly focused on P. aeruginosa PAO1 and PA14 model strains20,21 a still limited number of studies reported effects of QSI molecules on P. aeruginosa clinical isolates, especially MDR or XDR strains22. Diverse sources of QSI molecules that inhibit the virulence of P. aeruginosa have been described so far, both biogenic including plants, animals and bacteria23–25 or derived by chemical synthesis26.

**Results**

**Clinical isolate Delftia sp. 11304 produces QSI molecule(s).** Among 633 clinical isolates from a negative collection screened for quorum sensing inhibitory (QSI) activity (Supplementary Table 1), 19 strains (belonging to five genera) were selected as positive (Supplementary Table 2). Delftia sp. 11304 was selected as one of the most promising candidate with QSI activity. Growth phase dependence of the Delftia sp. 113014 QSI phenotype was determined by enumerating of colony forming units (CFU) at selected time points. Dependence of QSI activity from growth phase was determined at the same time points and expressed as numerical values obtained by measurement of inhibition zones of violacein production by Chromobacterium violaceum CV026.

![Figure 1](image.png) Growth phase dependence of QSI production by Delftia sp. 11304. Bacterial growth curve was determined by enumerating of colony forming units (CFU) at selected time points. Dependence of QSI activity from growth phase was determined at the same time points and expressed as numerical values obtained by measurement of inhibition zones of violacein production by Chromobacterium violaceum CV026.

with an attached fatty acyl side chain of 4 to 20 carbons13,14. Detection of AHLs occurs either directly by LuxR regulators within the cell, or by membrane-bound two-component histidine kinase-type proteins15,16. Interestingly, long-chain AHLs, C_{10}-AHL, C_{12}-AHL and C_{14}-AHL, were reported to bind to CviR AHL receptor in widely used reporter system Chromobacterium violaceum CV026 with the same affinity as C_{6}-AHL autoinducer, but they disrupt activity of CviR in terms of transcriptional activation17,18. Disruption of QS achieved by interference with QS signaling or interception of signal molecules is considered a key point for development of antibacterial and anti-disease strategies targeting pathogens like P. aeruginosa in medicine35. Indeed, interference with QS signaling by QSI-inhibitors (QSI) or interception of signal molecules by quorum quenching enzymes (QQE) results in a reduction of virulence regulated by QS. It is considered that QSI is a natural mechanism first developed either by QS-emitting organisms for the recycling or clearing of their own QS signals or by QSI organisms in the context of a competitive relationship with QS-signal-emitting organisms. Thus, bacteria that share ecological niche with P. aeruginosa during infections could be considered as promising producers of novel QSI molecules. Examples of the activity of QSI molecules, showing a successful reduction of siderophores, proteases, rhamnolipids secretion, as well as inhibition of biofilm formation have been previously documented. While those studies were mainly focused on P. aeruginosa PAO1 and PA14 model strains20,21 a still limited number of studies reported effects of QSI molecules on P. aeruginosa clinical isolates, especially MDR or XDR strains22. Diverse sources of QSI molecules that inhibit the virulence of P. aeruginosa have been described so far, both biogenic including plants, animals and bacteria23–25 or derived by chemical synthesis26.

**Delftia tsuruhatensis** strains have previously been investigated as plant growth-promoting rhizobacteria (PGPR) due to their production of siderophores which can mitigate iron limitation in soil27. However, recent findings pinpoint *D. tsuruhatensis* as an emerging pathogen associated with an increasing number of human infections28–30. It was previously published that *D. tsuruhatensis* could exhibit an anti-quorum sensing activity to *P. aeruginosa* quorum sensing systems, and authors identified a diisooctyl ester of 1,2 benzenedicarboxylic acid as an active compound31. Herein we analyzed inhibitory potential of *D. tsuruhatensis* 11304 clinical isolate against QS systems of *P. aeruginosa* MDR clinical isolate and characterized molecule(s) underlying this phenomenon.

**Whole genome sequencing.** Genomic DNA of the strain *Delftia* sp. 11304 was sequenced using Illumina HiSeq. 2500 platform (MicrobesNG, University of Birmingham, United Kingdom). A total of 6,616,336 sequences were generated from the genome. Assembly of reads resulted in 199 contigs with the largest contig 337425 and an average GC content of 66.78%. The contig dataset was used to determine the functional analysis. According
to the genomic sequence analysis Delftia sp. 11304 was identified as a Delftia tsuruhatensis species. A repertoire of different virulence factors and antibiotic resistance determinants were detected in D. tsuruhatensis 11304 genome (Supplementary Tables 4 and 5). Among the virulence factors presence of the genes encoding for different siderophores, pyoverdine, pyochelin and ornitobactin, as well as capsule and alginate could be pointed out. Additionally, within the D. tsuruhatensis 11304 resistome antibiotic resistance determinants such as genes encoding for efflux pumps of Resistance-Nodulation-Division (RND) superfamily (including: mexE, mexY, mexB, mexD, mexI, mexA, smeB, smeE, amrB and mdtB), beta-lactamases genes blaOXA-23 and blaLNBRA-13, as well as spirimycin resistance gene were identified as the most significant.

**Stability of the QSI molecule(s).** In order to determine whether D. tsuruhatensis 11304 QSI activity was based on proteinaceous or non-proteinaceous molecule activity, thermostability and resistance to proteinase were tested. Results showed that both overnight culture and cell-free supernatant retained QSI activity after the heat and proteinase K treatments (Fig. 2). Obtained results suggested that the QSI activity of D. tsuruhatensis 11304 was based on non-enzymatic quorum sensing inactivation mechanism.

**Ethyl acetate** *D. tsuruhatensis* 11304 extract exhibits QSI activity and inhibits biofilm formation of *P. aeruginosa* MMA83. Organic solvents with different polarity were used to optimize the extraction of the QSI molecule(s) from *D. tsuruhatensis* 11304 overnight culture supernatant. Only the ethyl acetate extract resuspended in DMSO was shown to be QSI active (Fig. 2), while methanol, chloroform and hexane extracts did not exhibit any QSI activity.

To exclude that the QSI effect of extract was a result of a bactericidal effect on the *P. aeruginosa* MMA83, we evaluated the antimicrobial activity of the QSI extract by microdilution method. As shown in Fig. 3, *D. tsuruhatensis* 11304 QSI ethyl acetate extract had no statistically significant effect on bacterial growth and biofilm formation. Additionally, we have demonstrated that the inhibition of *P. aeruginosa* MMA83 biofilm formation by *D. tsuruhatensis* 11304 was dose-dependent using crystal violet staining of biofilm biomass (Fig. 3). Indeed, biofilm formation decreased by 88.5% in the presence of 5 mg/ml QSI extract comparing to the control (**p < 0.001**). About 50% inhibition of biofilm formation was observed with a 0.156 mg/ml QSI extract (**p < 0.001**). The lowest tested concentration of QSI extract (0.038 mg/ml) showed decreasing activity of 21.9% in regard to positive control (*p < 0.05*). Treatment of *P. aeruginosa* MMA83 with the highest dose of DMSO used in experiment (0.5% v/v) had no statistically significant impact on bacterial growth and biofilm formation.

**D. tsuruhatensis** 11304 QSI extract prevents biofilm formation of *P. aeruginosa* MMA83, but it is unable to induce biofilm decomposition. Fluorescence microscopy has been used to visualize the effect of the QSI extract on *P. aeruginosa* MMA83 biofilm formation as well as on the decomposition of preformed biofilm. The captured images showed that *P. aeruginosa* MMA83 that grew in the absence of *D. tsuruhatensis* 11304 QSI extract formed a compact biofilm. In the presence of *D. tsuruhatensis* 11304 QSI extract biofilm was highly dispersed, with a clear decrease in the surface coverage and density of bacteria. Indeed, as shown in Fig. 4, minimal attachment of *P. aeruginosa* MMA83 cells could be observed in the treatment with a 5 mg/ml QSI extract (after 24 hours of incubation) (Fig. 4b).

**Figure 2.** *D. tsuruhatensis* 11304 QSI activity was determined using colorimetric agar well diffusion assay with Chromobacterium violaceum CV026 as an indicator strain. (a) The activity of untreated control of *D. tsuruhatensis* 11304 overnight culture (1) and cell-free supernatant (2). The heat treatment at 100 °C for 30 min (3, 4) and 60 min (5, 6). *D. tsuruhatensis* 11304 overnight culture (3, 5) and cell-free supernatant (4, 6) retained QSI activity and shown to be thermostable. Proteinase K treatment did not affect QSI activity of *D. tsuruhatensis* 11304 overnight culture (7) and cell-free supernatant (8). Ethyl acetate extract of *D. tsuruhatensis* 11304 exhibits QSI activity (9). Proteinase K and LB growth medium with 0.5% DMSO (10, 11) are used as negative controls. (b) Quantitative data of these experiments obtained by measurement of inhibition zones of violacein production by *Chromobacterium violaceum* CV026.
Interestingly, co-incubation of preformed *P. aeruginosa* MMA83 biofilm with the QSI extract (5 mg/ml) did not result in decomposition of the biofilm (Fig. 4d), although the biofilm architecture was less consistent and slightly different compared to the positive control (Fig. 4c), there were still visible living bacterial cells resembling the architecture observed in the positive control.
D. tsuruhatensis 11304 QSI extract shows synergistic effect with antibiotics. In order to investigate the clinical relevance of the QSI extract and its effectiveness in combination with clinically used drugs, the checkerboard method was assessed. Results indicate that the test strain *P. aeruginosa* MMA83 was susceptible to meropenem and gentamycin at very high used concentrations (MIC values 0.512 mg/ml and 4.096 mg/ml, respectively). MIC value of the *D. tsuruhatensis* QSI extract was 20 mg/ml. However, their combined application with the QSI extract showed a synergistic outcome for meropenem ($\sum\text{FIC} = 0.125$) and for gentamycin ($\sum\text{FIC} = 0.047$) (Table 1). DMSO (MIC value 145.45 mg/ml) had indifferent outcome for both of used antibiotics.

**Table 1.** The checkerboard method representing the effect of antimicrobials against *P. aeruginosa* MMA83. FIC – fractional inhibitory concentration, $\text{FIC} = \text{MIC combination}/\text{MIC alone}$. $\sum\text{FIC} = \text{FIC of antibiotic} + \text{FIC of QSI extract or DMSO}$.

| Antimicrobials | Pseudomonas aeruginosa MMA83 | MIC of each antimicrobial (mg/ml) | FIC | $\sum\text{FIC}$ | Outcome |
|---------------|-------------------------------|-----------------------------------|-----|----------------|---------|
|               | Alone                         | Combination                      |     |                |         |
| QSI extract   | 20                            | 1.25                             | 0.0625 | 0.125          | synergistic |
| Meropenem     | 0.512                         | 0.032                           | 0.0625 |               |         |
| DMSO          | 145.45                        | 145.45                           | 1    | 3              | indifferent |
| Meropenem     | 0.512                         | 1.024                           | 2    |               |         |
| QSI extract   | 20                            | 0.625                           | 0.03125 | 0.047          | synergistic |
| Gentamycin    | 4.096                         | 0.064                           | 0.0156 |               |         |
| DMSO          | 145.45                        | 145.45                           | 1    | 2              | indifferent |
| Gentamycin    | 4.096                         | 4.096                           | 1    |               |         |

**Figure 5.** Graph demonstrating the dose-dependent effect of the *D. tsuruhatensis* 11304 QSI extract on *P. aeruginosa* MMA83 virulence factors production. Black bars – production of elastase, white bars – production of rhamnolipid and gray bars – production of pyocyanin. Statistical significance was evaluated by Student’s *t*-test against control (0) without QSI extract (*p < 0.05, **p < 0.01, ***p < 0.001).

**D. tsuruhatensis 11304 QSI extract shows synergistic effect with antibiotics.** In order to investigate the clinical relevance of the QSI extract and its effectiveness in combination with clinically used drugs, the checkerboard method was assessed. Results indicate that the test strain *P. aeruginosa* MMA83 was susceptible to meropenem and gentamycin at very high used concentrations (MIC values 0.512 mg/ml and 4.096 mg/ml, respectively). MIC value of the *D. tsuruhatensis* QSI extract was 20 mg/ml. However, their combined application with the QSI extract showed a synergistic outcome for meropenem ($\sum\text{FIC} = 0.125$) and for gentamycin ($\sum\text{FIC} = 0.047$) (Table 1). DMSO (MIC value 145.45 mg/ml) had indifferent outcome for both of used antibiotics.

**D. tsuruhatensis 11304 QSI extract inhibits the virulence factors production of *P. aeruginosa* MMA83.** The impact of the QSI extract on the QS regulated virulence factors (elastase, rhamnolipid and pyocyanin) production in *P. aeruginosa* MMA83 was next investigated. A dose-dependent decrease in the production of all the analyzed virulence factors was observed (Fig. 5). The lack of the production of elastase, rhamnolipid and pyocyanin after treatment of the *P. aeruginosa* MMA83 strain with a 5 mg/ml *D. tsuruhatensis* 11304 QSI extract has been noticed. The treatment with a 0.156 mg/ml QSI extract resulted in about 40% inhibition of rhamnolipid and 50% inhibition of elastase and pyocyanin production compared to the positive control.

**D. tsuruhatensis 11304 QSI extract inhibits the expression of *P. aeruginosa* MMA83 QS systems.** In order to confirm that the above described effects of *D. tsuruhatensis* 11304 QSI extract on *P. aeruginosa* MMA83 virulence were caused by transcriptional inhibition of QS systems genes belonging to three *P. aeruginosa* QS networks (las – lasI, lasR; rhl – rhlI, rhlR; PQS – pqs, mvfR) were selected for RT-qPCR study. The obtained results revealed that the treatment of *P. aeruginosa* MMA83 with a 5 mg/ml QSI extract significantly decreased the mRNA levels ($***p < 0.001$) (Fig. 6). Relative expression of the genes coding for inducer synthases lasI and rhlI was 2.2 times lower, while the expression of transcriptional regulators lasR and rhlR was even more reduced (7.6 and 4.1 times lower, respectively) compared to the control. The most significant downregulation of transcription was observed in the case of the pqs gene (15 times lower), while the transcription of transcriptional regulator mvfR was 5.7 times lower compared to the control.
Determination of *D. tsuruhatensis* 11304 QSI extract composition by MALDI mass spectrometry.

To define the nature of the molecules composing the *D. tsuruhatensis* 11304 QSI extract, an aliquot of the sample was preliminarily purified on a thin-layer chromatography (TLC) silica gel. Separated spots were visualized, and then scratched off. The silica gel was put into a cotton-plugged column, and the sample was eluted from the gel by ethyl acetate, dried and then investigated by MALDI mass spectrometry. Several solvents were employed to dilute and analyze the QSI extract; nevertheless, the best resolved spectra were obtained by diluting the QSI extract directly in the matrix solution (see Methods section for details).

The positive ion MALDI mass spectrum, recorded in reflectron mode, is reported in Fig. 7a. The spectrum clearly indicated the presence of several [M + H]⁺ ions attributed to N-acyl homoserine lactones (AHLs) with chain lengths of C12 to C18. In particular, the main ion peak at *m/z* 368.3 was identified as N-octadecanoylhomoserine lactone (C₁₈-HSL). This was proven by the observation in the related positive ion MS² spectrum (Supplementary Fig. S3) of (i) the typical intense ion at *m/z* 102.06, common to all AHLs detected, relative to the protonated α-amino-γ-butyrolactone deriving from the cleavage of the carboxamide linkage between the acyl chain and the homoserine group; (ii) the counterpart acylium ion arising from the same cleavage (*m/z* 267.27) as well as (iii) the occurrence of a fragment originated from the loss of a water molecule from the [M + H]⁺ molecular ion (*m/z* 350.29) [22,33]. Similarly, other AHLs species with a shorter acyl chain have been identified at *m/z* 284.2 (C₁₂-HSL), *m/z* 312.3 (C₁₄-HSL) and *m/z* 340.3 (C₁₆-HSL). In traces,
against several different names, was previously identified as a QSI molecule, produced by *Delftia tsuruhatensis*, active of the quorum sensing systems. Diisooctyl ester of 1,2 benzenedicarboxylic acid, a compound that is called by *P. aeruginosa*.

In addition, the same concentration of C18-HSL statistically significant reduced transcription of the lasI gene in *P. aeruginosa* examined in this study belong to three *P. aeruginosa* QS pathways (las – lasI, lasR; rhl – rhlI, rhlR; PQS – mvfR). RT-qPCR data were normalized against the ribosomal gene *rpsL* as an internal control. (b) The changes in pyocyanin production after the treatment of *P. aeruginosa* MMA83 with a commercial C18-HSL. "C" on both graphs refers to the DMSO control. Student’s *t*-test was used to compare the differences between the control and experimental groups (*p* < 0.05, **p** < 0.01, ***p*** < 0.001).

C18-HSL reduces pyocyanin production and quorum sensing lasI gene expression in *P. aeruginosa* MMA83. In order to investigate QSI potential of C18-HSL its effect on expression of quorum sensing genes and pyocyanin production in *P. aeruginosa* MMA83 was analyzed. Small but statistically significant decrease in pyocyanin production by MMA83 grown in presence of 20 μM C18-HSL was observed (Fig. 8b). In addition, the same concentration of C18-HSL statistically significant reduced transcription of the lasI gene in MMA83, although the fold-change was small (Fig. 8a).

Discussion

Our understanding of *Delftia tsuruhatensis* has experienced a complete turnaround from the promising plant growth-promoting bacteria to emerging human pathogen predominantly isolated from respiratory specimens, blood and urine. There is a disparity between the growing importance of *Delftia* spp. isolates and genomic data from these species. Noticeably, *D. tsuruhatensis* genomic data are scarce since only the genome of *D. tsuruhatensis* MTQ3 is publicly available on NCBI. Although we could not speculate about the origin of the *D. tsuruhatensis* strain, which was characterized in this study (was it intrahospital, or an environmental strain introduced to the hospital by a patient), the presence of genetic determinants for the production of siderophores, capsule and alginate is a good indication of a possible virulent phenotype. Along with the genetic determinants of antibiotic resistance that were found within the *D. tsuruhatensis* genome, like beta-lactamases and RND pumps, that could contribute to the overall pathogenicity of this particular strain and could provide insight into the genetic armory of *D. tsuruhatensis* in general.

The reason why we included *Delftia* spp. in testing was our initial presumption that bacteria, like *D. tsuruhatensis* strain, which colonize the same tissues during infection of a host as *P. aeruginosa*, probably compete with *P. aeruginosa* by employing diverse mechanisms, including the interference of cell-to-cell signaling, i.e. quenching of the quorum sensing systems. Dissooctyl ester of 1,2 benzene dicarboxylic acid, a compound that is called by several different names, was previously identified as a QSI molecule, produced by *Delftia tsuruhatensis*, active against *P. aeruginosa* PAO1 QS. It is worth noticing that a contamination of laboratory samples by this compound was found when plastic tubes were used, since it is widely used as a plasticizer. In addition, our data showed that within the range of concentrations used in this study, bis (2-ethylhexyl) phthalate and dissooctyl phthalate were unable to interfere with *C. violaceum* CV026 quorum sensing system or with *P. aeruginosa* biofilm formation. Thus, our main aim was to characterize the QSI potential of *D. tsuruhatensis* against *P. aeruginosa* by using *C. violaceum* and a multidrug-resistant clinical isolate *P. aeruginosa* MMA83 as model systems. The *D. tsuruhatensis* ethyl acetate extract showed a remarkable ability to attenuate *P. aeruginosa* MMA83 QS, and to suppress its virulent phenotype by disabling its intrinsic ability to form a biofilm or to produce virulence factors such as elastase, rhamnolipid and pyocyanin.

Biofilm formation ability of *P. aeruginosa* MMA83 was significantly impaired by the *D. tsuruhatensis* extract, without affecting overall bacterial cell growth. This reduction in biofilm forming ability occurred in a dose-dependent manner, similarly as previously reported for other QSI extracts. However, the disruption of preformed biofilm was not achieved, which might result from biofilm matrix impermeability or possibility that it acts through QS inhibition. The production of extracellular virulence factors is of crucial importance for the invasion of host tissues, initial phases of biofilm formation and promoting virulence expression and it was shown
to be controlled by las and rhl QS systems\textsuperscript{38,39}. Our study demonstrated that the D. tsuruhatensis 11304 extract was highly capable of attenuating P. aeruginosa virulence, and thus possibly infection, by significantly reducing the production of these virulence factors. This anti-virulent effect of the D. tsuruhatensis 11304 extract was based on the inhibition on transcriptional level of las, rhl and pqs QS systems, indicating a possible therapeutic potential of the QSI molecule(s) produced by strain 11304.

Considering that classical isolates of P. aeruginosa often possess MDR or even XDR resistant phenotype, novel strategy for clinical treatment of infections caused by such isolates could be the combination of antibiotics with non-antibiotic bioactive compounds. It has been reported that different QS inhibitors caused a decrease in antibiotic resistance in P. aeruginosa PAO1 through synergistic effects with clinically used drugs\textsuperscript{40,41}. In our study, susceptibility of the MDR clinical isolate P. aeruginosa MMA83 to meropenem and gentamycin was enhanced by synergistic interactions of the D. tsuruhatensis 11304 QSI extract with antibiotics.

MALDI MS data showed that AHLs with chain lengths of C12 to C18 were present in the D. tsuruhatensis 11304 QSI extract, with the main ion peak attributed to N-octadecanoylhomoserine lactone (C\textsubscript{18}-HSL). Interestingly, peak at m/z 400.3 was noted and isolated as MS\textsuperscript{2} precursor ion furnishing important structural data. The corresponding MALDI MS\textsuperscript{2} spectrum (Fig. 7b), revealed the occurrence of the common ion at m/z 102.06 but also of an ion derived from the loss of water from the protonated molecular ion (m/z 382.29). Two peaks at m/z 281.25 and 263.24, matching with the loss of one and two water molecules respectively from the acyl chain, suggested the occurrence of two hydroxyl groups decorating the acyl chain. None of the fragments helped in understanding the position of the two hydroxyl moieties; nevertheless, according to literature data, it was assumed that a single hydroxyl group is placed at position 3 of the acyl chain whereas the position of the second remains to be defined. Therefore, a novel AHL species could be identified as composing the D. tsuruhatensis 11304 QSI-extract, specifically a dihydroxy-

\textsuperscript{-}octadecanoylhomoserine lactone (Fig. 7b). Although C\textsubscript{18}-HSLs were previously described\textsuperscript{42}, to our best knowledge this is the first report of a naturally occurring dihydroxy-

\textsuperscript{-}octadecanoylhomoserine lactone. Finding that commercial C\textsubscript{18}-HSL reduces las quorum sensing gene expression and pyocyanin production indicates its involvement in virulent potential of P. aeruginosa MMA83. This finding is of importance since it is known that, in certain bacterial species, long-chain AHLs interfere with the short-chain AHLs-mediated QS signaling\textsuperscript{17,18}. For example, C\textsubscript{10}-HSL fails to activate the CviR-dependent transcription in C. violaceum CV026, and yet functions as an antagonist in the presence of a native autoinducer C\textsubscript{6}-HSL. Additionally, it was shown that the lengthening of the AHLs’ acyl-tails in the CV026 model system reduces agonism and enhances antagonism due to the promotion of an inactive conformation of the CviR-ligand complex\textsuperscript{43}. It is tempting to speculate that the long-chain AHLs in the D. tsuruhatensis 11304 extract, among which a predominantly novel dihydroxy-

\textsuperscript{-}octadecanoylhomoserine lactone is present, could be responsible for the interference of the P. aeruginosa MMA83 quorum sensing system and mitigation of its virulence. An analogy could be drawn to the CV026 quorum sensing system since P. aeruginosa doesn’t produce AHLs with side chains longer than C12\textsuperscript{44}. Our findings could be also supported with observations made by Zhu et al\textsuperscript{45}, who showed that one of the strongest antagonists of TraR, which is a 3-oxo-C\textsubscript{6}-HSL-responsive transcriptional activator in Agrobacterium tumefaciens, has also hydroxyl residues placed at position 3 of the acyl chain. The authors of mentioned study also claimed that the length of the side acyl chain was one of key determinants affecting antagonistic properties of synthetic AHLs used in their model system. We should take into account that the observed QSI activity could not be attributed solely to C\textsubscript{18}-HSL but to an orchestrated activity of several molecules from the extract, due to limited effect of C\textsubscript{18}-HSL comparing to the effect of entire 11304 QSI extract. Thus, our further work will be focused on the separation and identification of all the components in D. tsuruhatensis 11304 ethyl acetate extract, and testing of their QSI activities independently.

**Methods**

**Bacterial strains and cultivation conditions.** Strain Delftia sp. 11304 from the collection of Laboratory for Molecular Microbiology (LMM), Institute of Molecular Genetics and Genetic Engineering (IMGGE), University of Belgrade, used in this study was isolated in a co-culture with Achromobacter xylosidoxidan 11304 from a cough swab\textsuperscript{46} in a tertiary type hospital in Belgrade, Serbia. Quorum sensing inhibition (QSI) activity of 633 clinical isolates from LMM collection was tested by using AHL biosensor strain Chromobacterium violaceum 026 (CV026), a mini-Tn5 mutant deficient in the AHLs synthase cvi\textsuperscript{7}. Pseudomonas aeruginosa MMA83, a New Delhi metallo-beta-lactamase-producing clinical strain from the laboratory collection (LMM, IMGGE) was used as a test strain\textsuperscript{47,48}. The bacteria were cultured aerobically in either Luria–Bertani (LB) broth medium or Mueller-Hinton medium (MH) at 37 °C for all isolates except C. violaceum CV026 which was grown at 30 °C. M9 medium (10 x M9 salts - Na\textsubscript{2}HPO\textsubscript{4}, KH\textsubscript{2}PO\textsubscript{4}, NaCl, NH\textsubscript{4}Cl; 20% glucose; 1 M MgSO\textsubscript{4}; 1 M CaCl\textsubscript{2}; dissolved in miliQ water) supplemented with 0.24% pyruvate was used for bacterial cultivation on a large scale for extraction of molecule(s) that interfere with quorum sensing system.

**QSI detection assay.** Initial screening for Delftia sp. 11304 QSI activity was performed with colorimetric agar well diffusion assay using Chromobacterium violaceum CV026 as an indicator strain. Overnight culture of C. violaceum CV026 (0.5% v/v) was inoculated in Luria–Bertani soft-agar (0.5% v/v) with addition of 5 μM N-(hexanoyl)-l-homoserine lactone – HHL (Sigma-Aldrich, Missouri, USA) and overlaid on LB agar (17% v/v). Wells were made in the solidified LB soft agar. Wells were filled with 50 μl of Delftia sp. 11304 culture and incubated for 24 h at 30 °C in order to test QSI activity\textsuperscript{49}. The absence of the violet color around the wells was considered as evidence of the inhibition of violacein pigment synthesis, and demonstrative of positive QSI activity.

QSI activity of Delftia sp. 11304 was monitored during different growth phases. Initially, 1% overnight culture was inoculated in fresh LB medium and grown at 37 °C, with aeration for 30 hours. Aliquots of bacterial culture were collected at different time points (0, 2, 4, 6, 8, 10, 12, 14, 16, 24 and 30 hours) and serial tenfold dilutions were
prepared for determination of CFU/ml number. In parallel, QSI activity was determined by colorimetric agar well diffusion assay where diameter of Q5 inhibition was measured. Experiment was done in triplicate.

Chemical compounds bis (2-ethylhexyl) phthalate and diisooctyl phthalate that possess the same molecular formula C_{24}H_{38}O_{4} (molecular weight 390.564 g/mol) and the same structural formula (diisooctyl phthalate) as a previously described quorum sensing inhibitor compound33 were used in order to confirm their QSI and antibacterial activity. Two-fold serial dilutions (started from 1024 µg/ml to 4 µg/ml) of bis (2-ethylhexyl) phthalate and diisooctyl phthalate solutions (Sigma–Aldrich) dissolved in ethanol (1:1 ratio) were tested for antibacterial activity on C. violaceum CV026 and P. aeruginosa MMA83, QSI activity (C. violaceum CV026) as well as biofilm forming effect (P. aeruginosa MMA83) using previously described methods.

Whole genome sequencing and genome analyses. Genomic DNA of Delftia sp. 11304 was sequenced using Illumina HiSeq by MicrobesNG service (MicrobesNG, IMI-School of Biosciences, University of Birmingham, Birmingham, UK). The quality of each sequencing library was assessed using FastQC45. IDBA-UD with multi k-mer mode outperformed the assembly using De Bruijn Graph methods51. In addition, unassembled reads were collected and assembled by Celera Assembler with the Best Overlap Graph–CABOG. Finally, raw reads were mapped to assembled scaffolds with Burrows Wheeler Aligner–BWA52. Delftia sp. 11304 was identified using the genome sequence and EzBioCloud 16S rRNA gene database available online at https://help.ezbiocloud.net/ezbiocloud-16s-database53.

Identification of genes coding virulence factors in the genome sequence was performed using virulence factor database (VFDB), an online resource for curating information about virulence factors of bacterial pathogens (http://www.mgc.ac.cn/VFs/main.htm)44 and Linux command line. Presence of the antibiotic resistance genetic determinants in the sequenced genome was determined by the publicly available database, The Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/)55 using bacterial pathogens line. Draft genome sequence of Delftia tsuruhatensis 11304 has been deposited at the NCBI GenBank database under accession number SMMJ00000000.

Stability of D. tsuruhatensis 11304 QSI molecule(s). In order to test the thermostability of D. tsuruhatensis 11304 QSI activity, overnight culture and cell-free supernatant were incubated at 100 °C for 30 and 60 min. Additionally, a potential proteinaceous nature of QSI activity was tested by treatment of D. tsuruhatensis 11304 overnight culture and cell-free supernatant with proteinase K (500 µg/ml) at 37 °C for 3 hours. Residual QSI activities after the heat or proteinase K treatments were evaluated by colorimetric agar well diffusion assay.

Preparation of the D. tsuruhatensis 11304 QSI extract. Bacterial culture D. tsuruhatensis 11304 (five liters) was cultivated in M9 medium supplemented with 0.24% pyruvate for 24 h, at 37 °C, aerobically. After centrifugation at 13,680 x g, at 4 °C, for 30 min, the collected supernatant was divided into four glass vessels and each of them was extracted with an equal volume of methanol, chloroform, ethyl acetate and hexane (Sigma–Aldrich) with vigorous shaking for 30 min at room temperature. Extracts were evaporated to dry using a vacuum rotary evaporator at 50 °C for ethyl acetate and 30 °C for methanol, chloroform and hexane (Buchi Rotavapor, R200, Fisher Scientific, Hampton, New Hampshire, US) and the dry mass was dissolved in dimethyl sulfoxide (DMSO) for further analyses56,57. In order to avoid contamination with phthalates from plasticware all experiments were done in glassware.

The effect of the D. tsuruhatensis 11304 QSI extract on P. aeruginosa MMA83 biofilm formation ability. In order to determine the minimal inhibitory concentration (MIC) of ethyl acetate QSI extract dissolved in DMSO on P. aeruginosa MMA83 planktonic cells, a 5 mg/ml QSI extract (following two-fold serial dilutions) was inoculated with 2 × 10^6 CFU/ml of the test strain using a 96-wells microdilution method. The MIC value was defined as the lowest concentration which inhibited bacterial growth. Controls were incubated with a concentration of DMSO equal to that used in the treatment (0.5% v/v). Number of CFU/ml was determined by plate serial tenfold dilutions after 24 h of incubation at 37 °C. The experiments were performed in sextuplicate and repeated three times. Afterwards, wells were washed to remove planktonic bacterial cells and stained with 0.1% (w/v) crystal violet (HiMedia Labs Pvt. Ltd., India)58. Biofilm formation was quantified by recording the absorbance at 595 nm using Plate Reader Infinite 200 pro (MTX Lab Systems, Austria).

The effects of the QSI extract on biofilm formation or decomposition of preformed biofilm were additionally visualized using fluorescent dye SYTO9 (TermoFisher Scientific, Massachusetts, USA) and propidium iodide (PI) (Sigma–Aldrich)59. For visualization of biofilms, P. aeruginosa MMA83 (2 × 10^5 CFU/ml) was cocultivated with a 5 mg/ml QSI extract in 24-well plates (Tissue Culture Plate, Sarstedt, Germany) which contained microscopic cover glass. After incubation (24 h, at 37 °C), the cells were washed three times with PBS and stained with SYTO9 (2.5 µM) and PI (2.5 µM), green and red fluorescent dyes, respectively. In order to test the decomposition of preformed biofilm in the presence of the QSI extract, P. aeruginosa MMA83 was cultivated for 24 hours, at 37 °C in 24-well plates, washed 3 times with PBS and, subsequently, treated with 5 mg/ml of QSI extract. After next 24 hours (48 hours in total), cells were washed and stained as previously described. Stained cells were visualized by fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, California, USA) under 20,000 × magnification. Untreated bacterial cells were used as a positive control.

QSI extract effect on antibiotic MIC values against P. aeruginosa MMA83. The combined effect of the D. tsuruhatensis 11304 QSI extract and selected clinically relevant antibiotics against P. aeruginosa MMA83 were assessed using 96-well plate microdilution method. Serial dilutions of meropenem (0.038, 0.125, 0.625, 3.125, 15.625, 78.125, 390.625 µg/ml) and gentamycin (0.038, 0.156, 0.76, 3.08 mg/ml) were cross-diluted with serial dilutions of QSI extract (20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.076 or 0.038 mg/ml) and DMSO (16, 8, 4, 2, 1, 0.5%). Different
concentrations of DMSO were expressed in mg/ml (16% corresponds with 145.45 mg/ml, etc.) in order to calculate final outcome for fractional inhibitory concentration. Plates (Tissue Culture Plate, Sarstedt, Germany) were filled with the bacterial suspension at a final density of 2 × 10⁵ CFU/ml. Cell density was recorded by OD₆₀₀ measurements using Plate Reader Infinite 200 pro (MTX Lab Systems, Austria) after 24 hours of incubation at 37 °C. The experiments were performed in triplicate and repeated two times.

The fractional inhibitory concentrations (FICs) were determined according to the previously described checkerboard method. The outcome was defined as a synergistic if the sum of two FICs (FIC of the antibiotic and FIC of the QQ extract) was ≤ 0.5; additive if 0.5 < ∑FIC ≤ 1; indifferent if 1 < ∑FIC < 4; antagonistic if ∑FIC > 4.

The effect of the *D. tsuruhatensis* 11304 QSI extract on the virulence factors production in *P. aeruginosa* MMA83. Elastase assay. Supernatants of overnight culture of *Pseudomonas aeruginosa* MMA83 were supplemented with two-fold diluted concentrations of the QSI extract (starting with 5 mg/ml, down to 0.038 mg/ml) and then mixed with Elastin-Congo red (Sigma-Aldrich) at a final concentration of 2 mg/ml. After 24 h of incubation at 37 °C, with shaking (180 rpm), the mixtures were centrifuged at 15,700 x g for 15 min, after which the elastase activity was measured at 495 nm using Plate Reader Infinite 200 pro (MTX Lab Systems, Austria) after 24 hours of incubation at 37 °C. The experiments were performed in triplicate and repeated two times.

Rhamnolipid assay. Rhamnolipid production was examined by acidification of supernatants with HCl (to pH 2) of the previously cultivated test strain (co-incubated with two-fold serial dilutions of the QSI extract or without the QSI extract). The absorbance was monitored spectrophotometrically at 570 nm by Plate Reader Infinite 200 pro (MTX Lab Systems, Austria).

Pyocyanin assay. *P. aeruginosa* MMA83 overnight culture (with two-fold dilutions of the QSI extract or without the QSI extract) was centrifuged at 15,700 x g for 15 min and pyocyanin was extracted from the supernatant using chloroform in a 1:2 ratio following the re-extraction of chloroform phase by 0.2 N HCl (3:1 ratio) according to the previously described method. The concentration of pyocyanin was evaluated by measuring the absorbance of the red top layer at 520 nm using Plate Reader Infinite 200 pro (MTX Lab Systems, Austria). Concentrations, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant, were determined by multiplying the optical density at 520 nm (OD₅₂₀) by 17.072.

Quantification of *P. aeruginosa* MMA83 quorum sensing genes expression by RT-qPCR. Reverse transcription-quantitative PCR (RT-qPCR) was performed to determine the expression levels of regulatory genes involved in quorum sensing. Primers used for the amplification of selected genes are listed in the Table 2. The total RNA was isolated from the test strain *P. aeruginosa* MMA83 (grown for 10 hours, at 37 °C) supplemented with the QSI extract (5 mg/ml) or without it by RNAeasy Mini Kit (Qiagen, Germany). The total RNA was then treated with DNase using an Ambion DNA-free kit (ThermoFisher, MA, US) and reverse transcribed by a Rever-tAid RT Reverse transcription Kit (ThermoFisher) according to the manufacturer’s instructions. Further amplification was achieved with KAPA SYBR Fast qPCR Kit (Kapa BioSystems, Wilmington, MA, USA) in a 7500 Real Time PCR System (Applied Biosystems, Waltham, MA, USA) under the following cycling conditions: incubation at 95 °C for 3 min and 40 cycles of 95 °C/15 s and 60 °C/1 min. RT-qPCR data was normalized against the ribosomal gene rpsL as an internal control following the 2⁻ΔΔCₜ method.

Isolation of QSI molecules by TLC. An aliquot (1 mg) of the *D. tsuruhatensis* 11304 QSI extract was dissolved in 100 µl of ethyl acetate and was applied to TLC (Silica gel 60, F₂₅₄ Aluminium sheets, Merck, Darmstadt, Germany) followed by developing with a mixture of chloroform and ethyl acetate in the ratio 5:4.

Separated spots were scratched off from the plate and the silica gel was put into a cotton-plugged column. The sample was then eluted from the silica gel with ethyl acetate.

| Gene   | Primer direction | Sequence (5’-3’) | Amplicon size (bp) | Source                  |
|--------|------------------|------------------|--------------------|-------------------------|
| lasI   | Forward          | 5-GCGTGGCTCAAGTGTTCAAGG-3 | 125                | This study              |
| lasR   | Reverse          | 5-GGAGTGGGGCCGATCGTCCGAG-3 | 127                | This study              |
| rhlII  | Forward          | 5-CCATCCGAAACCCGGCTACATGC-3 | 151                | This study              |
| rhlII  | Reverse          | 5-CTCCCCAGCGGCGATGGCTCCGAG-3 | 151                | This study              |
| rhlB   | Forward          | 5-GGGGCGGTTGTTGCGGCTTCCGG-3 | 143                | This study              |
| rhlB   | Reverse          | 5-GGTATGCCCTCCAGGCGGCCCTGG-3 | 143                | This study              |
| popA   | Forward          | 5-CCGGGACCTCAATCTCCTCCTCC-3 | 182                | This study              |
| popA   | Reverse          | 5-CGATAGCAGGGGGCTGCG-3   |                    | This study              |
| mvfR   | Forward          | 5-GTGGGGAGGGCTACAGGCTG-3  | 129                | This study              |
| mvfR   | Reverse          | 5-GATTGGCGGACCGCTTGTGAG-3 |                    | This study              |
| rpsL   | Forward          | 5-GCAACTATCAACCGAGCTGTTG-3 | 231                | This study              |
| rpsL   | Reverse          | 5-GCTGTGCTCTGCAAGTGTG-3   |                    | This study              |

Table 2. List of primers used for RT-qPCR.
MALDI MS and MS² of the D. tsuruhatensis 11304 QSI extract. The MS structural analysis was performed on an ABSCIEX TOF/TOF™ 5800 Applied Biosystems mass spectrometer equipped with an Nd:YLF laser (λ = 345 nm), with a pulse length of < 500 ps and a repetition rate of up to 1000 Hz. The dried sample obtained after TLC separation was redissolved either in acetonitrile, ethyl acetate, or directly in an α-cyano-4-hydroxycinnamic acid matrix solution (5 mg/ml) in acetonitrile/TFA 0.1% (70:30). The prepared QSI extract (0.5 µl) and matrix solution (0.5 µl), or 1 µl of the mixture were deposited on a stainless-steel plate and left to dry at room temperature. Each spectrum, acquired in positive ion mode, was a result of the accumulation of 2,000 laser shots, whereas 2,500 shots were summed for the MS² data acquisitions.

The effect of the commercial C18-HSL on the pyocyanin production and quantification of quorum sensing genes expression in *P. aeruginosa* MMA83. Commercial N-octadecanoylhomoserine lactone (C18-HSL) was purchased from Cayman Chemical (Tallin, Estonia) and resuspended in DMSO (0.5 mg/ml) according to manufacturer instructions. Concentrations of 5, 20 and 50 µM C18-HSL were used for evaluation of pyocyanin production and quantification of quorum sensing genes (listed in Table 2) expression in the presence of C18-HSL. Each concentration of C18-HSL was compared with control containing the exact volume of DMSO without C18-HSL to eliminate biological effect of DMSO in the experiment. Pyocyanin productions was expressed in µg/ml as described previously.56,84. Experiments were done as described above, in triplicate.

**Statistical analyses.** The statistical analyses and visualization were performed using GraphPad Prism software and SPSS 20.0 for Windows. The results are shown as means ± standard errors. The differences between control and experimental groups were compared using Student's t-test. A p value less than 0.05 was considered to be statistically significant.

Received: 14 April 2019; Accepted: 26 October 2019;
Published online: 11 November 2019

**References**

1. Araujo, D. et al. The independent contribution of *Pseudomonas aeruginosa* infection to long term clinical outcomes in bronchiectasis. *Eur Respir J.*, 51, 1701953, https://doi.org/10.1183/13993003.01953-2017 (2018).

2. Xie, J. et al. A 16-year retrospective surveillance report on the pathogenic features and antimicrobial susceptibility of *Pseudomonas aeruginosa* isolates from FAHJU in Guangzhou representative of Southern China. *Microb. Pathog.* 110, 37–41, https://doi.org/10.1016/j.micpath.2017.06.018 (2017).

3. Del Barrio-Tofino, E. et al. Genomics and susceptibility profiles of extensively drug-resistant *Pseudomonas aeruginosa* isolates from Spain. *Antimicrob Agents Chemother.* 61(11), E01589–E01517, https://doi.org/10.1128/AAC.01589-17 (2017).

4. Yayan, J., Ghebremedhin, B. & Rasche, K. Antibiotic resistance of *Pseudomonas aeruginosa* in pneumonia at a single university hospital center in Germany over a 10-year period. *PLoS ONE.* 10(10), e0139836, https://doi.org/10.1371/journal.pone.0139836 (2015).

5. Klockgether, J., Cramer, N., Weichmann, L., Davenport, C. F. & Tümmler, B. *Pseudomonas aeruginosa* genomic structure and diversity. *Front. Microbio.* 2, 150, https://doi.org/10.3389/fmicb.2011.00150 (2011).

6. Oliver, A. et al. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist Updat.* 21-22, 41–59, https://doi.org/10.1016/jдруп.2015.08.002 (2015).

7. Foerstlhiel J., Winstanley, C. & James, C. E. Novel therapeutic strategies to counter *Pseudomonas aeruginosa* infections. *Expert Rev. Anti-Infect Ther.* 10, 219–235, https://doi.org/10.1586/eri.11.168 (2012).

8. Marasoli, A. E. et al. Management of multidrug-resistant *Pseudomonas aeruginosa* in the intensive care unit: state of the art. *Expert Review of Anti- Infective Therapy.* 15(9), 861–871, https://doi.org/10.1080/14787210.2017.1367666 (2017).

9. Hentzer, M. et al. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J.* 22, 3803–3815, https://doi.org/10.1093/emboj/cdq366 (2003).

10. Hentzer, M. et al. Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology.* 148, 87–102, https://doi.org/10.1099/mic.0.20122287-148-1-87 (2002).

11. Pearson, J. P., Feldman, M., Iglewski, B. H. & Prince, A. *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. *Infect. Immun.* 68, 4331–4334, https://doi.org/10.1128/IAI.68.7.4331–4334.2000 (2000).

12. Lee, J. & Zhang, L. The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell.* 6, 26–41, https://doi.org/10.1007/s13238-014-0100-x (2015).

13. Williams, P., Winzer, K., Chan, W. C. & Camara, M. Look who’s talking: communication and quorum sensing in the bacterial world. *Philosophical Transactions of the Royal Society B.* 362, 1119–1134 (2007).

14. Arashida, N. et al. Identification of novel long chain N-acylhomoserine lactones of chain length C₉₋₁₂ from the marine phototrophic bacterium *Rhodovulum sulfidophilum*. *Bioscience, Biotechnology, and Biochemistry.* 82(10), 1683–1693, https://doi.org/10.1080/09108991.2018.1490168 (2018).

15. Fuqua, W., Winans, S. C. & Greenberg, E. P. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* 176, 269–275 (1994).

16. Freeman, J. A., Lilley, B. N. & Bassler, B. L. A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. *Mol. Microbiol.* 39, 139–149, https://doi.org/10.1046/j.1365-2958.2000.01684.x (2000).

17. McClean, K. H. et al. Quorum sensing and *Chromobacterium violaceum* violacinum: exploitation of violacine production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology.* 143, 3703–3711 (1997).

18. Swenn, L. R. et al. A Quorum-Sensing Antagonist Targets Both Membrane-Bound and Cytoplasmic Receptors and Controls Bacterial Pathogenicity. *Molecular Cell.* 35(2), 143–153, https://doi.org/10.1016/j.molcel.2009.05.029 (2009).

19. Grandclément, C., Tannière, M., Moréra, S., Dessaux, Y. & Faure, D. Quorum quenching: role in nature and applied developments. *EMS Microbiol. Rev.* 40(1), 86–116, https://doi.org/10.1093/emsec/fun038 (2016).

20. Diggle, S. P. et al. The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem. Biol.* 14, 87–96, https://doi.org/10.1016/j.chemb.2006.11.014 (2007).

21. Welsh, M. A. & Blackwell, H. E. Chemical genetics reveals environment-specific roles for quorum sensing circuits in *Pseudomonas aeruginosa*. *Cell. Chem. Biol.* 23, 361–369, https://doi.org/10.1016/j.chembiol.2016.01.006 (2016).

22. Guendouze, A. et al. Effect of Quorum Quenching Lacatonse in Clinical Isolates of *Pseudomonas aeruginosa* and Comparison with Quorum Sensing Inhibitors. *Front Microbiol.* 8, 227, https://doi.org/10.3389/fmicb.2017.00227 (2017).

23. Koh, C. L. et al. Plant-derived natural products as sources of anti-quorum sensing compounds. *Sensors (Basel).* 13(5), 6217–28, https://doi.org/10.3390/s130506217 (2013).
24. Yang, F. et al. Quorum quenching enzyme activity is widely conserved in the sera of mammalian species. FEBS Letters. 579, https://doi.org/10.1016/j.febslet.2005.05.060 (2005).

25. See-Too, W. S. et al. AIdP: a novel N-Acyl homoserine lactonase gene from Antarctic Planococcus sp. Scientific reports. 7, 42968, https://doi.org/10.1038/srep42968 (2017).

26. Aleksić, I. et al. Long-Chain 4-Aminoquinolines as Quorum Sensing Inhibitors in Serratia marcescens and Pseudomonas aeruginosa. ACS Chemical Biology. 12(5), 1425–1434, https://doi.org/10.1021/acschembio.6b01149 (2017).

27. Guo, H. et al. Comparative Genomic Analysis of Delftia tsuruhatensis MTQ3 and the Identification of Functional NRPS Genes for Siderophore Production. BioMed Research International. 3687619, 8, https://doi.org/10.1155/2016/3687619 (2016).

28. Tabak, O. et al. Port-related Delftia tsuruhatensis bacteria in a patient with breast cancer. New Microbiol. 36, 199–201 (2013).

29. Preiswerk, B., Ullrich, S., Speich, R., Bloomberg, G. V. & Hombach, M. Human infection with Delftia tsuruhatensis isolated from a central venous catheter. J Med Microbiol. 60, 246–8, https://doi.org/10.1099/jmm.0.021238-0 (2011).

30. Ranc, A., Dubourg, G., Fournier, P., Raoult, D. & Fenollar, F. Delftia tsuruhatensis, an Emergent Opportunistic Healthcare-Associated Pathogen. Emerg Infect Dis. 24(3), 594–596, https://doi.org/10.3201/eid2403.160938 (2018).

31. Singh, V. K., Mishra, A. & Jha, B. Anti-quorum Sensing and Anti-biofilm Activity of Delftia tsuruhatensis Extract by Attenuating the Quorum Sensing-Controlled Virulence Factor Production in Pseudomonas aeruginosa. Front Cell Infect Microbiol. 7, 337, https://doi.org/10.3389/fcimb.2017.00337 (2017).

32. Leipert, J., Treitz, C., Leippe, M. & Tholey, A. Identification and Quantification of N-Acyl Homoserine Lactones Involved in Bacterial Communication by Small-Scale Synthesis of Internal Standards and Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. J Am Soc Mass Spectrom. 28(12), 2538–2547, https://doi.org/10.1016/s1361-0177(17)-x (2017).

33. Morin, D., Grasland, B., Vallée-Réhel, K., Dufau, C. & Haras, D. On-line high-performance liquid chromatography-mass spectrometric detection and quantification of N-acylhomoserine lactones, quorum sensing signal molecules, in the presence of biological matrices. J Chromatogr. A. 1002, 79–92; https://doi.org/10.1016/s0021-9673(03)00730-1 (2003).

34. Nguyen, D. H., Nguyen, D. T. M. & Kim, E. K. Effects of di-(2-ethylhexyl) phthalate (DEHP) released from laboratory equipments. J Microbiol. Methods. 77, 374–388, https://doi.org/10.1016/j.mimet.2004.04.016 (2004).

35. Reid, A. M., Brougham, C. A., Fogarty, A. M. & Roche, J. J. An investigation into possible sources of phthalate contamination in the environment. Anal. Bioanal. Chem. 389, 651–660, https://doi.org/10.1007/s00216-007-1376-5 (2007).

36. Smolikas, M. et al. Comparative Genomic Analysis of Pseudomonas aeruginosa las and rhl operons. J. Bacteriol. 193, 5756–5767 (2001).

37. Lao, G. W., Hassett, D. J., Ran, H. & Kong, F. The role of pyocyanin in Pseudomonas aeruginosa infection. Trends Mol. Med. 10, 599–606, https://doi.org/10.1016/j.molmed.2004.10.002 (2004).

38. Ding, X. et al. Screening for novel quorum-sensing inhibitors to interfere with the formation of Pseudomonas aeruginosa biofilm. J. Med. Microbiol. 60(12), 1827–1834, https://doi.org/10.1099/jmm.0.024166-0 (2011).

39. Yang, Y. et al. A new quorum-sensing inhibitor attenuates virulence and decreases antibiotic resistance in Pseudomonas aeruginosa. Journal of Microbiology. 50, 987–993, https://doi.org/10.1007/s12275-012-2149-7 (2012).

40. Marketon, M. M. et al. Characterization of the Sinorhizobium melloti sinR/sinL locus and the Production of Novel N-Acyl Homoserine Lactones. Journal of Bacteriology. 184(20), 5686–5695, https://doi.org/10.1128/JB.184.20.5686-5695.2002 (2002).

41. Chen, G. et al. A Strategy for Antagonizing Quorum Sensing. Mol. Cell. 42(2), 199–209, https://doi.org/10.1016/j.molcel.2011.04.003 (2011).

42. Soukariieh, F., Williams, P., Stocks, M. J. & Câmara, M. Pseudomonas aeruginosa Quorum Sensing Systems as Drug Discovery Targets: Current Position and Future Perspectives. Journal of Med Chem. 61(23), 10385–10402, https://doi.org/10.1021/acs.jmedchem.8b00540 (2018).

43. Zhu, J. et al. Analogos of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraK protein of Agrobacterium tumefaciens. J. Bacteriol. 180, 5395–5405 (1998).

44. Filipic, B. et al. Uncovering Differences in Virulence Markers Associated with Achromobacter Species of CF and Non-CF Origin. Frontiers in cellular and infection microbiology. 7, 224, https://doi.org/10.3389/fcimb.2017.00224 (2017).

45. Jovic, B. et al. Emergence of NDM-1 Metallo-β-Lactamase in Pseudomonas aeruginosa Clinical isolates from Serbia. Ant Agents Chem. 55(8), 3929–3931 (2011).

46. Jovic, B. et al. The clinical isolate Pseudomonas aeruginosa MM83 carries two copies of the blaNDM-1 gene in a novel genetic context. Ant Agents Chem. AAC. 0232–12 (2013).

47. McLean, R. J., Pierson, L. S. & Fuqua, C. A simple screening protocol for the identification of quorum signal antagonists. J. Microbiol. Methods. 58, 351–360, https://doi.org/10.1016/j.mimet.2004.04.016 (2004).

48. Andrews, S. FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics. babraham.ac.uk/projects/fastqc (2010).

49. Peng, Y., Leung, H. C., Yiu, S. M. & Chin, F. Y. IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. Bioinformatics. 28(11), 1420–8, https://doi.org/10.1093/bioinformatics/bts174 (2012).

50. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics. 26(5), 589–95, https://doi.org/10.1093/bioinformatics/btp698. (2010).

51. Peng, Y. et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococcus. APMIS 115, 891–899, https://doi.org/10.1111/j.1600-0463.2007.apm.630.x (2007).

52. Stepanović, S. et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 115, 891–899, https://doi.org/10.1111/j.1600-0463.2007.apm.630.x (2007).

53. Milivojevic, D. et al. Biofilm-forming ability and infection potential of Pseudomonas aeruginosa strains isolated from animals and humans. Pathogens and Disease. 76(4), https://doi.org/10.1093/femspd/fty041 (2018).

54. Odds, F. C. Synergy, antagonism, and what the chequebook puts between them. J. Antimicrob. Chemother. 52, 1, https://doi.org/10.1093/jac/dko301 (2003).

55. Alipour, M. et al. Attenuation of Pseudomonas aeruginosa virulence factors and biofilms by co-encapsulation of bismuth–ethanedithiol with tobramycin in liposomes. Journal of Antimicrobial Chemotherapy. 65(4), 684–693, https://doi.org/10.1093/jac/dko306 (2010).
62. Rienzo, D. D., Kamalanathan, M. & Martin, P. Comparative study of the production of rhamnolipid biosurfactants by *B. thailandensis* E264 and *P. aeruginosa* ATCC 9027 using foam fractionation. *Process Biochemistry*. 51(7), 820–827, https://doi.org/10.1016/j.procbio.2016.04.007 (2016).

63. Saha, S., Thavasi, R. & Jayalakshami, S. Phenazine pigments from *Pseudomonas aeruginosa* and their application as antibacterial agent and food colourants. *Research Journal of Microbiol.* 3(3), 122–128, https://doi.org/10.3923/rjm.2008.122.128 (2008).

64. Essar, D. W., Eberly, L., Hadero, A. & Crawford, I. P. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *Journal of Bacteriology*. 172, 884–900 (1990).

65. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods*. 25, 402–408, https://doi.org/10.1006/meth.2001.1262 (2001).

Acknowledgements
This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia [Grant No. 173019]. MM was granted a Wood-Whelan Researcher Fellowship of International Union of Biochemistry and Molecular Biology. The Authors thank to Dr. Zorica Vasiljević, Dr. Dušan Milivojević, Dr. Vele Tešević and Dr. Goran Jovanović for their help.

Author contributions
B.J., M.K. and A.M. conceived the experiments, M.M., F.L., B.F., N.S., K.N. and L.S. conducted the experiments, B.J., M.K., A.M. and N.P. analyzed the results, F.D.L., N.P., M.K., A.M., B.F. and L.S. revised the manuscript. All authors reviewed and approved the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-019-52955-3.

Correspondence and requests for materials should be addressed to B.J.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019