Population dynamics and transcriptomic responses of *Pseudomonas aeruginosa* in a complex laboratory microbial community

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*Pseudomonas aeruginosa* tends to be among the dominant species in multi-species bacterial consortia in diverse environments. To understand *P. aeruginosa*'s physiology and interactions with co-existing bacterial species in different conditions, we established physiologically reproducible 18 species communities, and found that *P. aeruginosa* dominated in mixed-species biofilm communities but not in planktonic communities. *P. aeruginosa*'s H1 type VI secretion system was highly induced in mixed-species biofilm consortia, compared with its monospecies biofilm, which was further demonstrated to play a key role in *P. aeruginosa*'s enhanced fitness over other bacterial species. In addition, the type IV pili and Psl exopolysaccharide were required for *P. aeruginosa* to compete with other bacterial species in the biofilm community. Our study showed that the physiology of *P. aeruginosa* is strongly affected by interspecies interactions, and both biofilm determinants and type VI secretion system contribute to higher *P. aeruginosa*'s fitness over other species in complex biofilm communities.

INTRODUCTION

Biofilms can protect bacteria from hostile environments and also disperse bacteria to colonise in new niches. *Pseudomonas aeruginosa* is a model organism for studying biofilm formation in Gram-negative bacteria. The biofilm mode affords *P. aeruginosa* protection and renders it more difficult to control. Type IV pili (T4P), flagella, iron, extracellular DNA and Pel/Psl exopolysaccharides (EPS) are well known factors that contribute to the development of *P. aeruginosa* monospecies biofilms. The adaptability of *P. aeruginosa* to various environments, such as water, soil, sewage and plants, suggests its strong competitiveness for ecological niches. For example, *P. aeruginosa* is a dominant cultivable endophytic bacterium associated with *Pennisetum glaucum* (millet).

The broad-spectrum adaptability of *P. aeruginosa* also hints at its strong competitiveness against various other bacterial species in mixed-species microbial communities. Several studies have indicated that *P. aeruginosa* is able to gain fitness over competing species during its survival in either two-species or three-species co-cultures. For example, *P. aeruginosa* reduces the viability of *Staphylococcus aureus* and lyses S. aureus cells for iron repletion in planktonic co-cultures. In biofilms, *P. aeruginosa* inhibits *Staphylococcus epidermidis* growth and reduces the initial adhesion of *Agrobacterium tumefaciens* through the secretion of Psl and Pel exopolysaccharides and small diffusible molecules. *P. aeruginosa* decreases the swimming motility of *P. putida* and therefore inhibits its biofilm formation by producing 2-heptyl-3-hydroxy-4-quinolone. Quinolones secreted by *P. aeruginosa* can also reduce biofilm maturation for a broad range of bacteria. These studies demonstrate the competitiveness of *P. aeruginosa* in mixed-species interactions, from metabolic versatility to a strong biofilm formation capacity. Documented strategies in which *P. aeruginosa* exerts competition involve type VI secretion system (T6SS), generation of antibiotics, iron chelators, pyoverdine, rhamnolipid, pyocyanin, extracellular polysaccharide, fatty acid cis-2-decanoic acid, proteinase and other quorum-sensing system-regulated virulence factors.

*P. aeruginosa* co-exists with many different microbial species in natural settings. However, the main competitive advantage of *P. aeruginosa* in gaining fitness over other species within complex microbial communities remains unclear. Previous studies indicated that interspecies interactions in mixed-species microbial communities are complicated and involve both cooperation and competition. Therefore, monitoring the population dynamics of *P. aeruginosa* in complex microbial communities is important for elucidating how this interaction network is established, which may provide insights into its ecological role in complex multispecies biofilms.

Until recently, there has been a lack of robust tools to monitor the population dynamics in complex microbial communities. The high-throughput digital NanoString nCounter® system has originally been developed to profile the eukaryotic multiplexed gene expression with flexibility and sensitivity. However, this technology has never been reported for its utilisation on monitoring population dynamics in a complex microbial community. Here we modified the probes to achieve a new application in investigation microbial ecology and evaluated this technology in monitoring population dynamics in a complex laboratory mixed-
species microbial community with pairs of tailored probes directly hybridise to the 16S rRNA of each species instead of mRNA without any enzymatic reaction or DNA synthesis.\textsuperscript{29} We further investigated the transcriptome of \textit{P. aeruginosa} competition in this mixed-species community in both planktonic and biofilm modes of growth.

RESULTS

Establishment of the complex microbial community

Instead of proliferating as a single-species culture, \textit{P. aeruginosa} often grows as commensal species within mixed-species microbial communities containing many other bacterial species in natural environments as well as infection sites.\textsuperscript{31,32} We presumed that bacteria within the same region of colonisation would have the same chance to co-exist. To obtain a better understanding of \textit{P. aeruginosa} physiology in complex microbial communities and evaluate the NanoString nCounter\textsuperscript{®} system, we selected bacterial species that potentially co-exist with \textit{P. aeruginosa} in different environments and can be distinguished from other species by NanoString nCounter\textsuperscript{®} probes. Hence, 18 human pathogens/ environmental bacteria (Supplementary Table 1) were established as laboratory planktonic and biofilm communities as some of these species usually share the same niche in humans or in natural environments. Among these species, \textit{Acinetobacter baumannii}, \textit{Burkholderia cenocepacia}, \textit{Klebsiella pneumoniae} and \textit{S. aureus} can co-infect lungs with \textit{P. aeruginosa};\textsuperscript{33–36} \textit{Citrobacter amalonaticus}, \textit{E. coli} and \textit{Streptococcus galolyticus} can infect intra-abdominal infections; \textit{Chromobacterium violaceum}, \textit{S. aureus} and \textit{Stenotrophomonas maltophilia} can infect the skin or open wounds; \textit{Listeria monocytogenes} can colonise a host's gastrointestinal tract; \textit{P. putida} has been isolated from infected urinary tract and infected skin; \textit{Bacillus subtilis}, \textit{Elisabethkingia meningoseptica}, \textit{P. fluorescens} and \textit{Shewanella oneidensis} are widely distributed in natural environments; and \textit{P. syringae} and \textit{Xanthomonas campestris} have been associated with plants.\textsuperscript{37} The abovementioned settings all involve the potential co-existence of \textit{P. aeruginosa}. Both known and undiscovered natural environments are so complex and unexpected. This laboratory community may assist in understanding \textit{P. aeruginosa}'s interactions with complex microbial communities. In addition to investigating the ecological behaviour and physiological response of \textit{P. aeruginosa} in our model system, we also seek insights into how future studies on complex microbial communities can be shaped.

We used 10 times diluted tryptic soy broth (10% TSB) as the growth medium, which supports the growth of all the selected 18 bacterial species at 25°C (room temperature) (Supplementary Figure 1). Although \textit{A. baumannii}, \textit{C. amalonaticus}, \textit{C. violaceum}, \textit{E. meningoseptica}, \textit{E. coli} and \textit{K. pneumoniae} grew relatively faster than other species, and \textit{L. monocytogenes} and \textit{S. galolyticus} were unable to grow to a high cell density in 10% TSB; the growth curves indicated that 10% TSB was suitable for establishing the mixed-species microbial community, with \textit{P. aeruginosa} growth comparable to the majority of the species (Supplementary Figure 1).

In addition to the growth rate, the biofilm formation capacity of these bacterial species was also verified in monospecies culture. After 24 h of static incubation, \textit{A. baumannii} and \textit{K. pneumoniae} had the highest capacity to form biofilms (Supplementary Figure 2). Among the other 16 species, \textit{B. cenocepacia}, \textit{P. aeruginosa} and \textit{S. maltophilia} have similar biofilm formation capabilities (Supplementary Figure 2). However, \textit{B. subtilis}, \textit{C. violaceum}, \textit{E. meningoseptica}, \textit{P. putida}, \textit{P. syringae} and \textit{S. galolyticus} formed less biofilms in 10% TSB at 25°C (Supplementary Figure 2). In summary, the biofilm formation capacities of the 18 species differ, with no linear relationship with growth rates. We therefore established the planktonic and biofilm microbial communities by adjusting each species to an \textit{OD}_{600} value of 0.01 in 10% TSB medium as inocula.

Physiological reproducibility of the mixed-species microbial communities

Before investigating the population dynamics and physiology of \textit{P. aeruginosa} in the mixed-species communities, we employed RNA sequencing-based metatranscriptomics to examine the physiological reproducibility of both the planktonic and biofilm communities. The sequencing reads (7.8–9.2 million per sample) were assigned to 44 classified functional roles (SEED; 51.4 million reads) with unclassified reads in MEGAN6. Similar profiles of functional classifications of genes assigned to each community model by their functional properties were observed among three biological replicates (Fig. 1a), indicating that physiology is highly reproducible in our laboratory model for both planktonic and biofilm communities (Fig. 1a). The proportion of each small bar with different colours represents the ratio of different functional genes in communities. For example, expression level of ‘metabolite damage and its repair or mitigation’ genes is higher in biofilm communities than in planktonic communities. In contrast, expression levels of genes in ‘iron acquisition and metabolism’, ‘cell division and cell cycle’, ‘secondary metabolism’ are higher in planktonic communities than in biofilm communities (Fig. 1a). In addition, clear separation between the two clusters on the principal coordinates analysis (PCoA) plot confirmed that there is a significant difference between the metatranscriptome profiles of the planktonic community and the biofilm community. The close proximity among replicates of each community indicates the similarity in metatranscriptome profiles of the replicates (Fig. 1b).

Evaluation of NanoString nCounter\textsuperscript{®} 16S rRNA array for microbial population assay

Although the 16S rRNA gene amplicon sequencing approach is widely used to study the microbial population diversity, it has the drawback of PCR bias. A NanoString nCounter\textsuperscript{®} 16S rRNA array was developed to detect unique signals from complex hybridised samples at a single-molecule level by utilising special probes that captures target 16S rRNA sequence and omitting the PCR amplification step\textsuperscript{38} (Supplementary Table 1). To check the feasibility of the designed nCounter\textsuperscript{®} 16S rRNA array for detecting the 16S rRNA from different bacterial species in the total RNA mixture, we mixed the total RNA extracted from 14 individual bacterial species at different ratios in three different training groups (Table 1). These were subjected to nCounter\textsuperscript{®} 16S rRNA array analysis, with the total RNA from the planktonic and biofilm communities together in one batch. The percentage of each species in 16S rRNA reads according to nCounter\textsuperscript{®} 16S rRNA array analysis was compared with its ‘actual’ RNA percentage from the training groups to calculate the normalisation factors for each of the 14 species (Table 1). To verify this new technology, the \textit{P. aeruginosa} probe was tested against the background of other genera in the mixed-species community, with the other three \textit{Pseudomonas} species excluded. One Gram-positive probe was tested against the background of other genera with \textit{L. monocytogenes}'s absence. The nCounter\textsuperscript{®} 16S rRNA array yielded more reads to some species than accurate 16S rRNA readings, whereas the converse occurred for other species (Table 1). This trend highlighted the need to characterise each species by multiplying the normalisation factors (the variation fold) (Table 1), when nCounter\textsuperscript{®} 16S rRNA array is employed to reflect the true
percentage of each bacterial species in a mixed-species community.

P. aeruginosa is the dominant species in the mixed-species biofilm community. To maintain the reproducibility of this laboratory model, we used the same mixed-species community. Each individual species culture was normalised to an OD$_{600}$ reading of 0.01 in the mixture and cultivated for 1 d to establish the planktonic community, and 5 d to establish the biofilm community. Total RNA extracted from these communities were analysed by nCounter® 16S rRNA array.

In the 1-day-old planktonic community, the relative abundance of A. baumannii, C. violaceum and E. meningoseptica increased by twofold or more compared with their inocula, whereas the relative abundance of B. subtilis, P. aeruginosa, S. oneidensis, S. aureus, S. gallolyticus and X. campestris decreased to half or less compared with their inocula (Fig. 2 and Supplementary Table 2). Interestingly, the microbial population dynamics are quite different in the 5-day-old biofilm community compared with the planktonic community; the relative abundance of K. pneumoniae and P. aeruginosa increased to more than fourfold their initial inputs, whereas most of the other species decreased significantly (Fig. 2 and Supplementary Table 2). P. aeruginosa 16 S rRNA constituted ~32.62% of the total 16 S rRNA content, whereas K. pneumoniae constituted ~26.08% of that in 5-day-old biofilms (Fig. 2, and Supplementary Table 2) after normalisation factor adjustment (Table 1). The proportions of all bacterial species (including the four non-normalised ones) are summarised in Supplementary Figure 3.

To validate the observed microbial population dynamics, we used GFP-tagged P. aeruginosa to establish the 1-day-old planktonic community and the 5-day-old biofilm community as described above. The abundance of P. aeruginosa was detected and calculated using the fluorescence-proportion standard curve (Supplementary Figure 4). The fluorescence-proportion standard curve is preferred over the colony-forming unit counting approach.
owing to the difference in the growth rates and the colony sizes of these 18 bacterial species on plates, which contributes to the accuracy of dynamics population counting. Resuspended cells from both planktonic and biofilm communities were used for fluorescence-based proportion tests and next examined using confocal microscopy imaging (Fig. 3b, c). The results of the biological fluorescence-proportion standard curve-based experiment correlated well with the results of the normalised nCounter® 16S rRNA array analysis result; 'RNA percentage' shows proportion of single species total RNA in mixture. 'Fold' calculated from average values of ratio in Reads percentage to it in RNA percentage. Dash in RNA percentage indicates no RNA was mixed into the random mixture sample, i.e., some average fold values and standard deviations could not be calculated, also labelled with a dash.

Table 1. nCounter® 16S rRNA array feasibility detection

|                       | Reads percentage (%) | RNA percentage (%) | Fold (normalisation factor) |
|-----------------------|----------------------|--------------------|-----------------------------|
|                       | I        | II      | III     | I        | II      | III     |                  |
| A. baumannii          | 3.13     | 6.52   | 1.09    | 1.95     | 3.71   | 1.92    | 1.31 ± 0.65     |
| B. subtilis           | 0.18     | 0.07   | 0.06    | 0.11     | 0.10   | 0.10    | 1.00 ± 0.61     |
| B. cenocecapia        | 3.88     | 0.54   | 1.01    | 1.93     | 1.76   | 9.55    | 0.81 ± 1.04     |
| C. violaceum          | 16.94    | 3.43   | 3.05    | 21.48    | 9.77   | 10.60   | 0.48 ± 0.27     |
| C. amalonaticus       | 7.56     | 6.36   | 0.10    | 11.45    | 20.84  | -       | 0.48 ± 0.25     |
| E. coli               | 2.10     | 2.50   | 0.97    | 1.88     | 6.83   | 3.71    | 0.58 ± 0.47     |
| E. anophelis          | 0.87     | 0.00   | 0.00    | 0.41     | -      | -       | 2.10 ± -        |
| K. pneumoniae         | 6.90     | 0.29   | 0.11    | 40.48    | -      | -       | 0.17 ± -        |
| L. monocytogenes      | 0.01     | 0.02   | 0.03    | -        | -      | -       | -               |
| P. aeruginosa         | 23.28    | 47.95  | 72.63   | 6.53     | 29.69  | 57.95   | 2.14 ± 1.24     |
| P. fluorescens        | 0.44     | 0.92   | 1.46    | -        | -      | -       | -               |
| P. putida             | 2.22     | 4.98   | 6.13    | -        | -      | -       | -               |
| P. syringae           | 0.01     | 0.01   | 0.01    | -        | -      | -       | -               |
| S. oneidensis         | 0.28     | 0.35   | 0.24    | 1.24     | 2.25   | 2.44    | 0.16 ± 0.06     |
| S. aureus             | 13.24    | 5.18   | 2.09    | 3.73     | 3.40   | 1.84    | 2.07 ± 1.30     |
| S. maltophilia        | 0.47     | 0.01   | 0.00    | 0.59     | -      | -       | 0.79 ± -        |
| S. gallolyticus       | 2.67     | 10.34  | 0.91    | 0.58     | 4.25   | 0.58    | 2.86 ± 1.54     |
| X. campestris         | 15.82    | 10.52  | 10.10   | 7.65     | 17.40  | 11.32   | 1.19 ± 0.78     |

Three different training groups were named 'I', 'II' and 'III' in this table. Numbers indicate the ratio of one species in the mixed group with unit %. 'Reads percentage' is nCounter® 16 S rRNA array analysis result; 'RNA percentage' shows proportion of single species total RNA in mixture. 'Fold' calculated from average values of ratio in Reads percentage to it in RNA percentage. Dash in RNA percentage indicates no RNA was mixed into the random mixture sample, i.e., some average fold values and standard deviations could not be calculated, also labelled with a dash.

Both the H1 type VI secretion system and biofilm formation determinants contributed to the fitness of P. aeruginosa in the biofilm community. As P. aeruginosa constituted up to 32.62% of the biofilm community in contrast to only 3.81% in the planktonic community (Supplementary Table 2), we hypothesised that the important biofilm determinants are likely to contribute to the dominating competence of P. aeruginosa over other species in the biofilm community. To test this hypothesis, we performed a transcriptomics analysis comparing the gene expressions in monospecies biofilm inocula with that of the mixed-species biofilm inocula as illustrated as a heat map (Fig. 4a). Further clustering of the transcriptome by principle component analysis revealed that the P. aeruginosa...
maintained a distinct physiology in monospecific and mixed-species biofilms (Fig. 4b). Based on the negative binomial test with an adjusted P value cutoff of 0.05 and a log2-fold change cutoff of 1,239 genes were upregulated and 171 genes were down-regulated in *P. aeruginosa* cells in the mixed-species biofilm compared to those in monospecific biofilms (Supplementary Table 3).

Among the significantly upregulated genes of *P. aeruginosa* in the mixed-species biofilm, at least 36 belonged to the T6SS (Table 2), which accounted for 15% of all the upregulated genes. Most of these belong to the Hcp secretion island I (H1) T6SS, whereas some are located in the Hcp secretion island II (H2) T6SS. However, there were only a few genes that belong to H3-T6SS. The ClpV protein is the energy source of T6SS39; the expressions of *clpV1, clpV2* and *clpV3* belonging to three different sub-type T6SS in *P. aeruginosa* were all upregulated in the mixed-species biofilm (Table 2). To validate the transcriptomic analysis results, the Δ*clpV1, ΔclpV2* and Δ*clpV3* mutants of *P. aeruginosa* were constructed with a GFP tag and thereafter formed biofilms with the other 17 species. Interestingly, only the Δ*clpV1* mutant was greatly impaired in terms of fitness to dominate in the mixed-species biofilms; when ClpV1 was complemented into this mutant with multiple copies, the complementation strain showed higher fitness to dominate in the mixed-species biofilm community than both wild-type PAO1 and the deletion mutant (Fig. 5). This finding suggests that the H1-T6SS has a significant effect on fitness gains for *P. aeruginosa*, enabling it to outcompete other species in the mixed biofilm community (Fig. 5). In addition, confocal images confirmed that parental strain PAO1 gains higher fitness over the other bacterial species than its isogenic H1-T6SS defect mutant.

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**Fig. 3** Proportions of *P. aeruginosa* in the mixed-species microbial communities. **a** Circular dots indicate three biological replicates of *P. aeruginosa* normalised proportions in both the planktonic and biofilm communities as measured by NanoString nCounter® 16S rRNA array; the square dots show three biological replicates of *P. aeruginosa* proportions in mixed-species microbial communities as calculated by fluorescence-based proportion test. The lines show the average values and standard deviation. Comparison was performed by t test with p < 0.05. **b** Confocal images of the resuspended 24 h-incubated mixed-species planktonic microbial community containing GFP-tagged *P. aeruginosa*. **c** Confocal images of the resuspended 5-day mixed-species biofilm community containing GFP-tagged *P. aeruginosa*. Green cells show the GFP-tagged *P. aeruginosa*, red cells show all of other cells stained with Syto62. Scale bar represents 10 µm.
ΔclpV1 in the 18 species biofilm communities (Supplementary Figure 5c and 5d). H1-T6SS contains seven effectors to be secreted into its competitor, which are termed Tse1-7.40,41 Both Tse1 and Tse3, which can lyse the target cells by degrading their peptidoglycan,26 were upregulated in the complex microbial biofilm community (Table 2). Furthermore, the expression of the gene encoding the H1-T6SS delivery-dependent proteins VgrG1 was also increased (Table 2).

In addition to T6SS, the *P. aeruginosa pelF, pelG, flgC* and *flgD* genes, which are important for biofilm formation in monospecies
| Locus tag | Gene name | Fold change | p value | Product | Pathway |
|-----------|-----------|-------------|---------|---------|---------|
| PA0070    | tagQ1     | 2.66        | 1.20E-09| TagQ1   | H1-T6SS |
| PA0074    | ppkA      | 2.06        | 1.77E-04| Serine/threonine protein kinase PpkA | H1-T6SS |
| PA0077    | icmF1     | 2.43        | 2.68E-07| IcmF1   | H1-T6SS |
| PA0079    | tssK1     | 2.06        | 4.11E-07| TssK1   | H1-T6SS |
| PA0082    | tssA1     | 2.27        | 5.28E-04| TssA1   | H1-T6SS |
| PA0083    | tssB1     | 5.34        | 1.81E-23| TssB1   | H1-T6SS |
| PA0084    | tssC1     | 5.24        | 1.51E-31| TssC1   | H1-T6SS |
| PA0085    | hcp1      | 4.82        | 1.20E-52| Hcp1    | H1-T6SS |
| PA0086    | tagJ1     | 2.43        | 4.84E-04| TagJ1   | H1-T6SS |
| PA0088    | tssF1     | 2.85        | 6.79E-07| TssF1   | H1-T6SS |
| PA0089    | tssG1     | 2.62        | 8.73E-04| TssG1   | H1-T6SS |
| PA0090    | clpV1     | 4.96        | 1.52E-21| ClpV1   | H1-T6SS |
| PA0091    | vgrG1     | 3.53        | 1.57E-12| VgrG1   | H1-T6SS |
| PA0092    | tsi6      | 2.4         | 8.41E-04| Tsi6    | H1-T6SS |
| PA0097    | PA0097    | 2.48        | 7.21E-05| Hypothetical protein | |
| PA0099    | PA0099    | 3.61        | 2.72E-15| Type VI effector protein | |
| PA0100    | PA0100    | 2.86        | 8.24E-15| Hypothetical protein | |
| PA0260    | tle3      | 2.62        | 2.67E-17| Tle3    | H2-T6SS |
| PA0261    | PA0261    | 2.34        | 2.26E-05| Hypothetical protein | |
| PA0263    | tssC2     | 3.06        | 7.18E-18| TssC2   | H2-T6SS |
| PA0265    | tssJ2     | 2.8         | 5.26E-08| TssJ2   | H2-T6SS |
| PA0266    | clpV2     | 2.36        | 1.79E-10| ClpV2   | H2-T6SS |
| PA0267    | sfa2      | 2.61        | 5.56E-08| Sfa2    | H2-T6SS |
| PA0268    | hfa2      | 2.74        | 3.11E-09| Hfa2    | H2-T6SS |
| PA0269    | lp2       | 3.3         | 2.74E-11| Lip2    | H2-T6SS |
| PA0270    | tssJ2     | 2.8         | 5.26E-08| TssJ2   | H2-T6SS |
| PA0271    | dotU2     | 2.27        | 1.18E-05| DotU2   | H2-T6SS |
| PA0272    | icmF2     | 3.21        | 1.74E-14| IcmF2   | H2-T6SS |
| PA0273    | tse1      | 2.86        | 1.62E-03| Tse1    | H1-T6SS |
| PA0274    | tsi1      | 2.81        | 4.93E-04| Tsi1    | H1-T6SS |
| PA0275    | tssB3     | 3.78        | 6.97E-22| TssB3   | H3-T6SS |
| PA0276    | tssC3     | 3.75        | 1.81E-37| TssC3   | H3-T6SS |
| PA0277    | hcp3      | 3.08        | 1.35E-18| Hcp3    | H3-T6SS |
| PA0278    | tssG3     | 2          | 1.03E-04| TssG3   | H3-T6SS |
| PA0279    | clpV3     | 2.11        | 1.07E-08| ClpV3   | H3-T6SS |
| PA0280    | vgrG4a    | 2.17        | 1.02E-03| VgrG4a  | H2-T6SS |
| PA0281    | tse3      | 2.54        | 6.03E-04| Tse3    | H1-T6SS |
| PA0282    | rhlA      | 2.35        | 4.99E-07| Rhamnosyltransferase chain A | Quorum sensing |
| PA0283    | rhlB      | 2.17        | 8.81E-07| Rhamnosyltransferase chain B | Quorum sensing |
| PA0284    | lasA      | 2.42        | 1.64E-08| LasA protease precursor | Quorum sensing |
| PA0285    | lasB      | 2.72        | 3.03E-12| LasB    | Quorum sensing |
| PA0286    | lasl      | 2.43        | 1.13E-14| Autoinducer synthesis protein Lasl | Quorum sensing |
| PA0287    | pqsH      | 2.09        | 3.42E-05| Probable FAD-dependent monooxygenase | Quorum sensing |
| PA0288    | flgC      | 2.08        | 1.14E-05| Flagellar basal-body rod protein FlgC | Flagella assembly |
| PA0289    | flgD      | 2.26        | 2.60E-10| Flagellar basal-body rod modification protein FlgD | Flagella assembly |
| PA0290    | pelF      | 2.44        | 2.40E-03| PelF | Extracellular polysaccharide biosynthesis |
| PA0291    | pelG      | 2.82        | 1.84E-03| PelG | Extracellular polysaccharide biosynthesis |
Quorum sensing is not required for the fitness of *P. aeruginosa* in the mixed-species biofilm community

Quorum-sensing systems play important roles in biofilm formation both in vitro and in vivo.  
*P. aeruginosa* is able to outcompete other microbial species by releasing quorum-sensing-regulated virulence factors, such as rhamnolipid, elastase, exotoxins, pyocyanin and pyoverdine.  
However, the expression of the *P. aeruginosa* quorum sensing-related genes, such as *lasA*, *lasB*, *rhlA*, *rhlB* and *pqsH*, were downregulated in this biofilm community compared with the monospecies biofilm (Table 2), suggesting that quorum sensing is not important in contributing to the fitness of *P. aeruginosa* in the mixed-species biofilm community.  
Interestingly, deleting the *lasB* or *mvr* quorum-sensing genes in *P. aeruginosa* even increased this species’ fitness over other species in the mixed-species biofilms, compared to the PAO1 wild-type (Fig. 5). This could possibly be owing to *P. aeruginosa* H1-T6SS being repressed by LasR and MvrR.

**FIG. 5** *P. aeruginosa* population dynamics in the mixed-species biofilm community affected by H1-T6SS and biofilm formation determinants. Each column shows the population dynamics of GFP-tagged *P. aeruginosa* parental strain PAO1 and the knockout mutants in the mixed-species microbial planktonic (black) and biofilm (grey) communities at different heights with the standard deviation of three biological replicates (dots). The asterisk indicates statistical significance by t test with p < 0.005.

Cultures, were also upregulated in the mixed-species biofilms (Table 2). We next investigated the impact of the classic monospecies *P. aeruginosa* biofilm determinants, e.g., Pil and Psl EPS, T4P, T4R and flagella, on the fitness of *P. aeruginosa* in the mixed-species biofilms. The *P. aeruginosa* deletion mutants ΔpslBCD, ΔpilA, ΔpilA or ΔfliM were used to cultivate mixed-species biofilms with the other 17 bacterial species respectively. The Psl polysaccharide and T4P were required for *P. aeruginosa* to predominate in the mixed-species biofilm community (Fig. 5). Psl was particularly essential for bacterial colonisation in the mixed-species biofilms, and ΔpslBCD was completely outcompeted by other species in the biofilms (Fig. 5), even though its planktonic growth was similar to that of the wild-type PAO1 strain (Supplementary Figure 6).

**DISCUSSION**

Our study reported on the establishment of physiologically reproducible laboratory planktonic and biofilm communities and their use in investigating how *P. aeruginosa* competes with multiple other bacterial species. We tracked the population dynamics and profiled the transcriptomes of the established communities and showed that *P. aeruginosa* maintains distinct transcriptomes in complex microbial communities compared to its monospecies cultures (Fig. 4). The H1-T6SS, Psl and T4P were found to be the key components for *P. aeruginosa* to gain fitness over other species in the mixed-species biofilm communities (Fig. 5 and Table 2). These pathway-involved genes, such as *clpV1* (68-fold), *pilA* (17.1-fold) and the *pslBCD* operon, were all significantly upregulated in the microbial biofilm community (*P. aeruginosa* is the dominant species) compared with the planktonic cultures (*P. aeruginosa* is not the dominant species). Our results highlight the biological significance of biofilm formation for *P. aeruginosa* to gain fitness in complex microbial communities, and we confirmed that H1-T6SS is a powerful weapon for *P. aeruginosa* interspecies competition within the ecological niche in bacterial communities. Confocal imaging analysis for dual-species biofilms also showed that parental strain PAO1 had a higher population than the H1-T6SS-defective mutant ΔclpV1 when competing against *A. baumannii*, *C. violaceum*, *C. amalonaticus* and *S. maltophilia* while *E. coli* as control (Supplementary Figure 5e–5n). Our findings on *P. aeruginosa* in mixed-species microbial communities are in accordance with previous studies in pure culture or two-species consortia and suggest that Psl, T4P and H1-T6SS are potential targets to control or eliminate *P. aeruginosa* from complex microbial biofilm communities, such as microbial biofilms on medical devices.

In addition, we demonstrated the sensitivity of the single molecule detection technique, NanoString nCounter® 16S rRNA array, in monitoring the population dynamics in complex bacterial communities. Our results showed that the accuracy of this technique depends on the specificity and affinity of the probes, which need to be normalised during the calculation of population dynamics.

*P. aeruginosa* can dominate airway infection microbiota and wound communities after the administration of antibiotics, suggesting that *P. aeruginosa* has a competitive physiology in mixed-species microbial communities. Our transcriptomic analysis showed that two of the primary mechanisms for *P. aeruginosa* maintaining this competitive advantage are H1-T6SS and biofilm formation. This differs from most previous reports in which many quorum-sensing-regulated virulence factors, such as elastase, PQS, pyoverdine and pyocyanin, were used by *P. aeruginosa* to kill other bacteria. The reasons for this difference may be as follows: (1) *P. aeruginosa* las and pqs systems repress H1-T6SS to compete with other bacteria; (2) quorum-sensing-signalling molecules and virulence factors might function as signals that induce aggressive phenotypes from bacterial competitors as well as hosts via cross-talk, which in turn arrests the growth of *P. aeruginosa*; (3) the high production of quorum-sensing-regulated virulence factors might consume a great deal of energy to reduce the division of *P. aeruginosa* cells or even trigger autolysis phenotypes; and/or (4) the continued fresh medium stream of a flow-tube biofilm cultivation system might wash away the secreted quorum-sensing signals and virulence factors and thus reduce the efficacy of quorum sensing. Thus, the cell-contact based T6SS might be the most efficient mechanism for *P. aeruginosa* to outcompete other species. This is supported by our data that loss of ClpV1 in H1-T6SS will decrease the competitive capacity of *P. aeruginosa* in our laboratory model (Fig. 5). Recently, Allsopp et al. has shown that all three T6SSs in *P. aeruginosa* are more highly expressed at 25°C than at 37°C, however, expressed H2-T6SS and H3-T6SS did not affect *P. aeruginosa* competition capacity as much as H1-T6SS at 25°C in our experiment (Fig. 5).

H1-T6SS is well known for its roles in competitiveness and pathogenicity in *P. aeruginosa*. In addition to H1-T6SS, Psl and T4P are critical components of *P. aeruginosa* fitness in the mixed-species biofilm community (Fig. 5). Our result is in accordance with previous research showing that secreted protein A from *S. aureus*...
can inhibit P. aeruginosa biofilm formation by binding to PsI and T4P. In mixed-species biofilm communities, three P. aeruginosa's most upregulated genes, PA3661, PA2205 and PA1395, are encoding small lipoproteins (Supplementary Table 3). Wood et al. reported P. aeruginosa's small lipoproteins genes upregulation under cell wall stress, which is regulated by sigma factor AlgU. However, expression of algU changed less than twofold in our study, suggesting that P. aeruginosa may undergo AlgU-independent stress response in the mixed-species biofilm communities. One limitation of our current study is that we have not elucidated whether P. aeruginosa employs AlgU-dependent stress response in the mixed-species biofilm communities. In addition, further study is required to validate whether the T6SS, PsI and T4P are required by P. aeruginosa to outcompete other species in vivo. Because the amounts of bacteria from in vivo samples are usually low and insufficient for transcriptomic analysis, suitable probes for the NanoString nCounter® array can be explored to examine the population dynamics and gene expression of P. aeruginosa from the in vivo communities. Moreover, K. pneumonia is the secondary dominant species in our biofilm co-cultures but not in planktonic co-cultures (Fig. 2), and it constantly co-exists with P. aeruginosa in vitro and in vivo. Thus, it would be interesting to ascertain its survival mechanism in future investigations.

METHODS

Bacterial strains, media and growth conditions

The bacterial species used in this study are listed in Supplementary Table 1. All strains were revived on TSB agar plates and subsequently in TSB liquid medium at 30 °C. Mixed-species communities were cultivated in 10% TSB medium at 30 °C. To construct P. aeruginosa gene knockout mutants and green fluorescent protein (GFP)-tagged strains, AB minimal medium supplemented with 10 mM citric acid supplemented with appropriate antibiotics was used to select P. aeruginosa. E. coli was grown in LB medium at 37 °C. In total, 60 µg/ml of ampicillin was used to select P. aeruginosa, whereas 15 µg/ml of gentamicin, 10 µg/ml of chloramphenicol and 100 µg/ml of ampicillin were used to select E. coli, when appropriate.

Growth assay and static biofilm cultivation

Overnight cultures of each bacterial species were diluted to OD600 of 0.01 in 10% TSB, and 150 µl diluted culture were loaded into 96-well microtitre plates. The plates were incubated in an infinite M200PRO multimode microplate reader (Tecan Schweiz AG, Männedorf, Switzerland) at 25 °C, and the OD600 of each well was measured and recorded hourly. A 10% TSB blank medium was used as a negative control, and its reading has been subtracted from all the culture readings to plot the growth curves for each species.

To examine the static biofilm-forming capacities of each species, bacterial cultures were prepared using the same procedures as for the growth assay. After overnight cultivation, the planktonic cells were discarded from the microtitre plate, and the wells were washed three times using tap water to remove any residual planktonic cells. A 180 µl volume of 0.1% crystal violet (CV) solution was loaded into each well to stain the biofilm for 15 min. The excess stain in the wells was washed out with tap water three times. Finally, 180 µl of 30% acetic acid was loaded into each well to dissolve CV from the stained biofilm. The optical intensity at OD550 was measured by an Infinite M200PRO multimode microplate reader to quantify the biofilms formed by each bacterial species.

Cultivation of the mixed-species planktonic and biofilm communities

Overnight cultures of the 18 individual bacterial species were diluted in 10% TSB medium and normalised to the same ratio (OD600 of each species is ~0.01) to form the initial inoculum for both planktonic cultures in shake flask and biofilm cultures in flow-tube reactors. The planktonic cultures were shaking with 200 rpm at 25 °C. The biofilm cultivation was started from initial inoculum attachment to Masterflex silicone tube; then fresh 10% TSB medium was continually pumped to flow through these attached cells in silicone tube at 4 ml/h at 25 °C. The 4 ml/h flow will supply enough nutrients for biofilm maturity and take away the planktonic cells in silicone tube. The 24-hour-old planktonic cultures and 5-day-old biofilm cultures were harvested for population dynamic or transcriptomic analysis.

RNA preparation for sequencing

The bacterial cells were first treated with RNA Protect Reagent (Qiagen®, Germany) to maintain the integrity of RNA. The total RNA was extracted from these bacterial cells using a miRNeasy Mini Kit (Qiagen®, Germany) with modifications. A Turbo DNA-free Kit (Thermo Fisher Scientific®, Lithuania) was used to remove genomic DNA contaminants from total RNA. RNA contamination was assessed with a Qubit® dsDNA High Sensitivity assay (PicoGreen dye) and a Qubit® 2.0 Fluorometer (Invitrogen®, Austria) according to manufacturer's instructions. Ribosomal RNA was depleted with a Ribo-Zero rRNA removal Kit (illumina, USA). The integrity of the total RNA was assessed with an Agilent TapeStation System (Agilent Technologies, UK).

The double-stranded complementary DNAs (cDNAs) were reverse-transcribed using a NEBNext RNA first and second strand synthesis module (NEB®, USA). cDNAs were subjected to Illumina’s TruSeq Stranded mRNA protocol. The quantitated libraries were then pooled at equimolar concentrations and sequenced on an Illumina HiSeq2500 sequencer in rapid mode at a read-length of 100 bp paired-ends.

NanoString nCounter® population analysis

One nanogram of purified total RNA from the different bacterial cultures was analysed using a NanoString nCounter® Gene Expression CodeSets platform (NanoString Technologies, Inc., United States) with the customised CodeSets for our selected bacterial species (Supplementary Table 1), according to the manufacturer’s protocol, to obtain raw counts. The quality control assessment and raw counts were normalised and analysed using nSolver® analysis software version 2.6 (NanoString Technologies, Inc., United States). The samples were analysed using the manufacturer-recommended default parameters. The geometric mean was selected for negative control subtraction; If the normalisation factors were outside the range 0.3–3, the normalised factor and the flag lane were computed by the geometric mean. The experiments were performed in triplicate, and the results are presented as the means ± S.D.

Transcriptomic analysis

The accession number for RNA-seq is SRP128411. The PAO1 genome (NC_002516) was used as the reference for P. aeruginosa transcriptomics analysis, with annotation from the Pseudomonas Genome Database (http://www.pseudomonas.com/). The RNA-Seq raw data were analysed using the “RNA-Seq and expression analysis” application in CLC genomics Workbench 10.0 (QiAGEN). The total gene reads from CLC genomics Workbench 10.0 were subjected to the DESeq2 package for statistical analysis by using R/Bioconductor. A hierarchical clustering analysis was performed with a negative binomial test using the DESeq2 package. The heatmap.2 package was used to draw a heat map for the differentially expressed genes of P. aeruginosa cells with fold change larger than two and an adjusted p value smaller than 0.05. The principal component analysis plot was generated in R/Bioconductor.

The adapter-trimmed and assembled RNA sequences of the mixed-species microbial community were used as metatranscriptomics data for analysis. The sequences of the mixed-species communities grown in biofilm and planktonic modes were aligned against the NCBI non-redundant protein database using DIAMOND with default settings. The output aligned gene reads in DAA format were uploaded and ‘megenised’ in MEGAn6.11.1 with a minimum bit-score of 50 and a top percentage of 25. Functional analyses of these aligned gene reads were performed using SEED classifications. A PCoA was plotted to cluster the samples based on functions. The functional analysis results are illustrated in stacked bar charts.

Fluorescence-based P. aeruginosa population assay

To develop a fast and economical P. aeruginosa population dynamics assay in the mixed-species cultures, a single copy of a GFP tag was inserted into the P. aeruginosa chromosome by using a mini-Tn7-Gm-gfp transposon. The other 17 bacterial species without P. aeruginosa were collectively used
as a background control. The GFP fluorescence readings of both planktonic and biofilm cultures, with gradient proportion of GFP-tagged *P. aeruginosa* derived strains, were recorded using a Tecan infinite M200PRO microplate reader with excitation wavelength 485 nm and emission wavelength 535 nm. The fluorescence-proportion standard curves of each tested strain were generated from these GFP readings (Supplementary Figure 4). The proportions of *P. aeruginosa* derived strains in communities were calculated using these fluorescence-proportion standard curves. All of coefficient of determinations ($R^2$) of these linear regression lines are larger than 0.97, suggesting that the fluorescence-based population assay is a reliable method to detect *P. aeruginosa* proportions in our mixed-species communities.

Constructions of *P. aeruginosa* gene deletion and complementation mutants

The upstream fragment of target gene was amplified by primer-1 and primer-2, the downstream fragment of the target gene was amplified by primer-3 and primer-4 (Supplementary Table 4), and then both fragments were fused into a pK18-Gm-mobscB plasmid$^{24}$ and transformed into the *P. aeruginosa PAO1* wild-type strain to delete the target gene as previously described.$^{12}$ PCR and sequencing were used to confirm the selected mutant strains (Supplementary Table 5). To complement the *P. aeruginosa clpV1* mutant, a DNA fragment from the clpV1 upstream 22 bps to the downstream 31 bps was amplified by primers clpV1-up and clpV1-down (Supplementary Table 4) and double-digested using BamHI and HindIII. The purified fragment was ligated with the pUCP22 vector transcribed by a lacZ promoter,$^{13}$ which was digested by the same restriction enzymes to construct the pUCP22-clpV1 complementary plasmid. After sequencing verification, the pUCP22-clpV1 plasmid was transformed into the *P. aeruginosa ΔclpV1* mutant by electroporation to construct a clpV1 complementation strain.

Confocal microscopy imaging

*P. aeruginosa* PAO1-GFP-containing planktonic cultures and biofilm cultures (the same samples as those used for the fluorescence-based population assay) were stained with 5 μM SYTO$	extsuperscript{TM}$ 62 Red fluorescent nucleic acid stain (molecular probe) for 15 min to stain all cells before 5 μl of each sample was aliquoted onto a glass slide and covered with a cover slip. For supplementary figure 5, 5 d biofilm cells were scraped from silicone tube into imaging chamber to stain all cells with SYTO$	extsuperscript{TM}$ 62 Red fluorescent nucleic acid stain, because silicone tube is too thick for directly confocal imaging. The samples were visualised and confocal images acquired using confocal laser scanning microscopy (CLSM; Zeiss LSM 780, Carl Zeiss, Germany) with a 63 × 1.40 DICII objective. Green and red fluorescence were observed upon 488 nm and 633 nm excitation wavelengths, respectively.

**DATA AVAILABILITY**

The data that support the findings of this study are available from the corresponding author upon reasonable request. The accession number for RNA-seq is SRP128411. Supplemental information is available at npj Biofilms and Microbiomes's website. The article was previously published as a preprint online.

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**AUTHOR CONTRIBUTIONS**

Y.C. designed and carried out experiments, interpreted results and wrote the manuscript. L.Y. designed the project and revised the manuscript. J.K.H.Y. helped with the confocal microscopy imaging and biofilm bioreactors. Z.C. performed metatranscriptomic analysis. Y.D. L.Z. and Y.D. helped to revise the manuscript.

**ADDITIONAL INFORMATION**

**Supplementary information** accompanies the paper on the npj Biofilms and Microbiomes website (https://doi.org/10.1038/s41522-018-0076-z).

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