Transcriptional profiling of *Pseudomonas aeruginosa* biofilm life cycle stages reveals dispersal-specific biomarkers

Xavier Bertran i Forga[1,2], Kathryn E. Fairfull-Smith[3,4,] Jilong Qin[1,5*], and Makrina Totsika[1,2*]

[1]Centre for Immunity and Infection Control, School of Biomedical Sciences, Queensland University of Technology, Brisbane, QLD, Australia

[2]Max Planck Queensland Centre, Queensland University of Technology, Brisbane, QLD, Australia

[3]School of Chemistry and Physics, Queensland University of Technology, Brisbane, QLD, Australia

[4]Centre for Materials Science, Queensland University of Technology, Brisbane, QLD, Australia

[5]Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia

*correspondence: Jilong Qin (j.qin@uq.edu.au), and Makrina Totsika (makrina.totsika@qut.edu.au)

Key Words: *Pseudomonas aeruginosa,* biofilm maturation, biofilm dispersal, RNA-seq, transcriptomics, c-di-GMP, dispersal biomarkers, biofilm life cycle.

Abstract

Bacteria exhibit two lifestyles: planktonic free-floating individual cells or sessile multicellular aggregates known as biofilms. The biofilm lifecycle is characterised by three distinct stages: attachment, maturation and dispersal. Specific signals govern each stage triggering responses that spatially and temporally regulate bacterial attachment to a surface, synthesis of extracellular matrix components and their subsequent degradation. Characterising these signals is therefore a valuable approach to develop novel antibiofilm therapies. Here, we used the model biofilm-forming bacterium *Pseudomonas aeruginosa* PAO1 to characterise the transcriptional profiles of each stage of the biofilm life cycle: attachment, biofilm maturation and spontaneous dispersal. We report that surface attachment was accompanied by the upregulation of genes comprising the mechanosensor Pil-Chp, whereas biofilm maturation characterised with the upregulation of genes involved in Pel polysaccharide synthesis, *siaD* and PA4396 diguanylate cyclases as well as *pipA, fimX* and PA5442. In contrast, dispersing cells upregulated genes responsible for the biosynthesis of alginate, rhamnolipid, and extracellular nucleases (*eddA*, *eddB*), as well as the transcriptional regulator of dispersal *amrZ*. Additionally, genes encoding the quorum sensing dispersal molecule cis-2-decenoic acid (*dspS* and *dspI*), canonical phosphodiesterases (*nbdA* and *rbdA*) and nine other c-di-GMP–related enzymes were also upregulated during dispersal. Our comprehensive analysis of transcriptional profiles associated with different biofilm stages allowed us to define a subset of fourteen genes as biomarkers whose transcription was largely upregulated during biofilm dispersal. Our study therefore provides benchmarking stage-specific transcriptional profiles for *P. aeruginosa* biofilms in closed culture systems, which led to the identification of a dispersal fingerprint marking the onset of dispersal.

Importance

Biofilm infections by *P. aeruginosa* are a major medical challenge due to the increased tolerance to antimicrobials displayed by bacteria living in sessile communities, which are forfeited during spontaneous biofilm dispersal. Attachment, biofilm maturation and dispersal conform the main stages

of a dynamic process known as the biofilm lifecycle. However, the global responses governing the transitions through each stage remain understudied. Here, we combine live microscopy and biomass quantification to track the progression of *P. aeruginosa* cultures through the three main stages of the biofilm lifecycle. We show that cells from each stage recapitulated canonical stage-specific transcriptional responses and identify a set of dispersal-specific biomarkers. These biomarkers may offer a practical tool for rapidly screening dispersal-inducing compounds, aiding in the discovery of the next generation of antibiofilm therapeutics.

Introduction

Pseudomonas aeruginosa is a highly adaptable Gram-negative opportunistic pathogen, which survives in hostile environments by forming cell aggregates embedded in a protective extracellular matrix primarily composed of exopolysaccharides, extracellular DNA (eDNA) and structural proteins [1]. These aggregates, known as biofilms, exhibit increased tolerance to desiccation and host defences compared to their planktonic counterparts [2]. *P. aeruginosa* biofilms are associated with chronic infections, which exhibit high tolerance to a broad range of clinically employed antimicrobials [2].

In laboratories, *P. aeruginosa* biofilms are frequently studied using a variety of culture systems such as closed culture systems (i.e. microtiter plates) or open-flow systems (i.e. flow cells), in which three stages are exhibited: attachment, biofilm maturation and dispersal and each stage is characterised by distinct molecular mechanisms [3]. During attachment, cells transition from the motile to sessile lifestyle. This process is mediated by the mechanosensors Pil-Chp and Wsp which lead to the upregulation of genes involved in the synthesis of matrix components and fimbrial adhesins such as CupA [4–6]. During biofilm maturation, the components of the matrix are progressively secreted into the extracellular space. As biofilms grow and mature, environmental cues and intracellular signals may accumulate, such as the fatty acid signal cis-2-decenoic acid, eventually triggering spontaneous dispersal responses [7]. This phenomenon is characterised by biofilm-residing single cells regaining motility and resuming the planktonic lifestyle, during which they escape the biofilm matrix by hydrolysing its components [8]. This is achieved by the upregulation of glycosyl hydrolases such as

79 PslG and PelA for exopolysaccharide Psl and Pel degradation [8–10], and extracellular endonucleases
80 EndA, EddA and EddB [8–10]. This process is influenced by the intracellular concentration of the master
81 regulator cyclic-di-GMP (c-di-GMP) [6], which is controlled by a family of proteins harbouring
82 diguanylate cyclase domains (DGCs; possessing a GGDEF motif) and phosphodiesterase domains
83 (PDE; possessing a HD-GYP or EAL motifs), which respectively synthesise and degrade it [11–14].

84 The transition between the stages of the biofilm life cycle is driven by distinct signalling pathways. To
85 characterise them, transcriptomics has been applied to biofilms treated with biofilm-dispersing agents
86 such as NO donors or carbon sources, which primarily captured immediate transcriptional changes
87 triggered by these compounds leading to an abrupt interruption of the biofilm maturation process [15–17].
88 However, the progression through the biofilm stages is a dynamic process [18], urging a comprehensive
89 characterisation of transcriptional profiles between each stage. Therefore, we here performed RNA
90 sequencing to compare the currently understudied transcriptional variations across cells undergoing
91 attachment, biofilm maturation, and dispersal [33]. Subsequently, by tracking the transcription patterns
92 of genes throughout the three life cycle stages, we identified a subset of biomarkers for biofilm
93 dispersal.

94

95 # Results

96 **Microtiter plate-grown *P. aeruginosa* displays the three defined biofilm life stages**

97 We previously showed that the biofilm life cycle of the model organism *Pseudomonas aeruginosa*
98 PAO1 in microtiter plates consisted of the following phases: 1) the progressive attachment of biomass
99 to the surface, reaching maximum biomass between 2-8 h in a process known as biofilm maturation
100 and, 2) a marked reduction in attached biomass known as the biofilm dispersal stage [18]. To further
101 characterise how these stages occur in closed culture systems such as microtiter plates, we quantified
102 the biofilm biomass (Fig 1A) and performed microscopy analysis simultaneously on forming and
103 dispersing biofilms at different time points to capture morphological changes during biofilm

formation (Fig 1B, Fig S1). Using this approach, we observed that the attachment stage occurs within 2 h of inoculation (Fig 1A and B; orange), during which cells adhered to the surface as a monolayer, with a culture density (OD_{600}) of ~0.1 and a low level of biomass (stained by crystal violet with OD_{550} of ~2.0). At 4-8 h post-inoculation, cells transitioned to the biofilm maturation stage (Fig 1A and B; blue), organised in voluminous scattered clusters that grew over time and produced multicellular biofilm structures (Fig 1B, Fig S1), as observed by the peak biofilm biomass staining quantified at the 8 h time point (Fig 1A). This build-up in biomass occurred between the early (OD_{600} ~0.3) and the late-logarithmic phase (OD_{600} ~1.5). Beyond 8 h post-inoculation and at a higher culture density (OD_{600}>1.5), biofilms biomass rapidly decreased. This phenomenon was characterised by disassembled structures leaving clearly distinguishable biofilm remnants attached to the well surface and a loss of 77% of accumulated biomass, (Fig 1A and B, Fig S1; green). Collectively, our data reflects that cells grown in closed culture systems synchronically transition through distinctive phases culminating in spontaneous biofilm dispersal. Therefore, these systems can effectively recapitulate the stages of the biofilm life cycle originally described in open-flow culture systems (flow cells) [3,19,20].

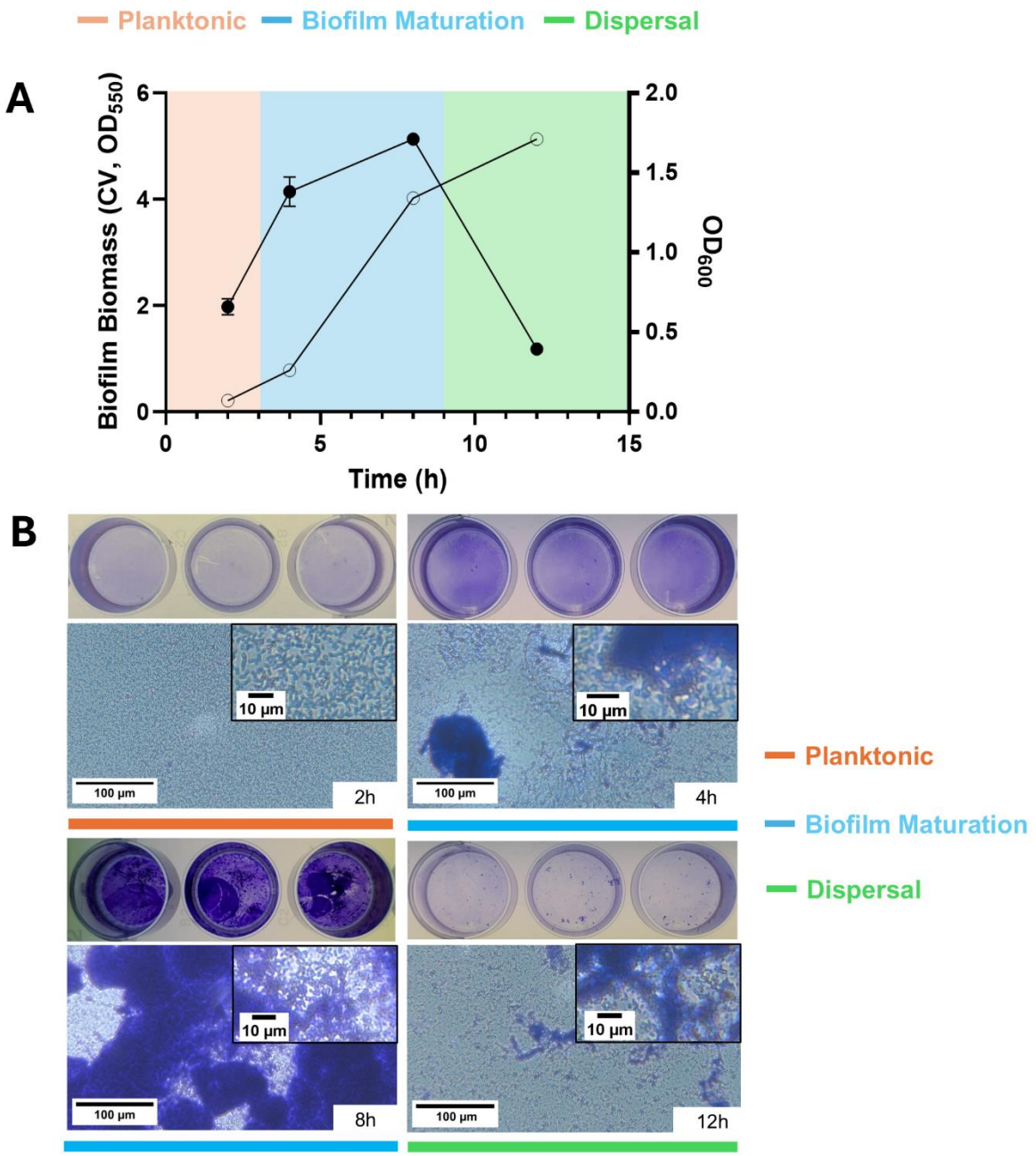


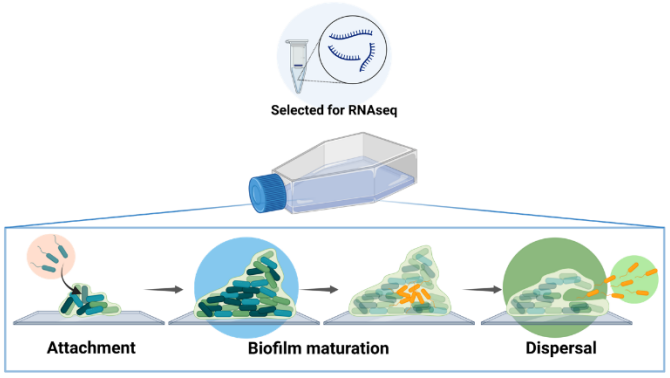
Figure 1. Biofilm formation and dispersal kinetics of *P. aeruginosa* PAO1. (A) *P. aeruginosa* PAO1 biofilm biomass and culture turbidity over 12 h. PAO1 cultures were seeded on 24-well plates and biofilm biomass was quantified at different time points by crystal violet (CV) staining (●). Culture density was simultaneously monitored by OD_{600} (○) with means ± SD shown. Data are representatives for at least 3 biological replicates for crystal violet staining and 1 biological replicate for OD_{600}. **(B)** Photographic and microscopic images of the crystal violet-stained biofilms at each stage of the biofilm life cycle. Crystal-violet-stained microscopic images of *P. aeruginosa* biofilm cultures in 24-wells. Images are representative of at least

3 biological replicates. Orange: attachment stage. Blue: biofilm maturation stage. Green: dispersal stage. This colour coding scheme is used throughout the article to differentiate the three biofilm stages.

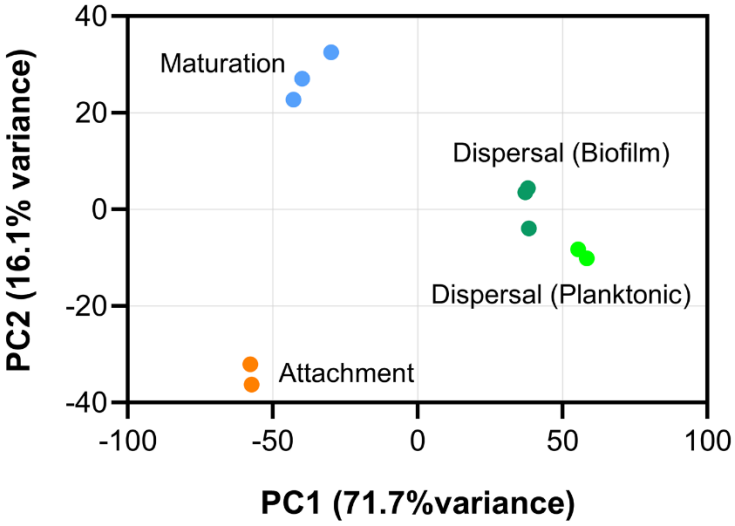
Transcriptional profiles recapitulate canonical pathways of attachment, maturation and dispersal

Having observed that *P. aeruginosa* cells grown in microtiter plates synchronically undergo the three defined stages of the biofilm life cycle, we performed RNA sequencing of *P. aeruginosa* during attachment, biofilm maturation and dispersal to characterise stage-specific transcriptional profiles (Fig 2A). Differential transcription analysis using a negative binomial test (P-value ≤ 0.01; Log_2 fold-change $\geq |1|$) identified a total of 3361 genes with significant transcriptional changes across all three comparisons: i) Attachment vs. Biofilm Maturation; ii) Biofilm Maturation vs. Dispersal; and iii) Attachment vs. Dispersal) (Table S1). Principal component analysis (PCA) further confirmed that cells at each stage formed distinct clusters, indicating discrete transcriptional profiles were associated with attachment, maturation, and dispersal (Fig 2B). Specifically, in cells undergoing biofilm maturation 540 genes were upregulated, and 405 genes were downregulated compared to attaching cells, whereas 1,240 genes were upregulated and 1,207 genes downregulated in maturing biofilms relative to dispersing bacterial cells (Fig 2C).

A


Selected for RNAseq
Attachment
Biofilm maturation
Dispersal

B


Maturation
Dispersal (Biofilm)
Dispersal (Planktonic)
Attachment
PC2 (16.1% variance)
PC1 (71.7%variance)
40
20
0
-20
-40
-100
-50
0
50
100

C

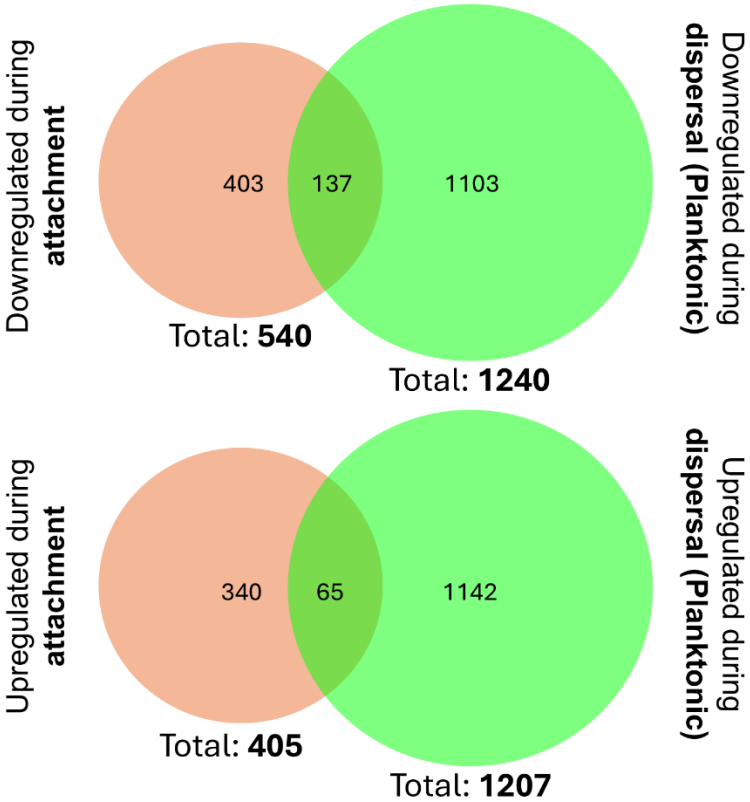

Downregulated during attachment
Downregulated during dispersal (Planktonic)
403
137
1103
Total: 540
Total: 1240
Upregulated during attachment
Upregulated during dispersal (Planktonic)
340
65
1142
Total: 405
Total: 1207

Figure 2. Transcriptional profiles of different stages of the biofilm life cycle. (A) Schematic depicting RNA sample collection for sequencing. During attachment, the free-swimming phase was collected to represent cells rapidly transitioning from motile to sessile. During biofilm maturation, surface-attached cells were collected, and cells from both phases were collected during the dispersal stage. **(B)** Principal component analysis of samples collected at distinct stages of the biofilm life cycle (attachment – orange; biofilm maturation – blue; the planktonic phase of biofilm dispersal – light green; and the biofilm phase of biofilm dispersal – dark green). Each biological replicate of a group is represented as a dot of the same colour. The normalised reads of all genes were then extracted for each sample and transformed into PC loadings with ClustVis. Values for PC1 and PC2, accounting for the largest variance percentage are represented in the plot. **(C)** Venn diagrams showing differentially transcribed genes in the planktonic phases of attachment and dispersal (relative to biofilm maturation). Downregulated (top) and upregulated (bottom) genes at each stage relative to biofilm maturation are represented separately. Overlapping areas indicate genes with consistent transcriptional changes relative to biofilm maturation stage.

Building on these global transcriptional variations and to further validate our datasets, we mined our RNA-seq data for previously reported stage-specific changes in the transcription of genes previously associated with attachment, biofilm formation or dispersal. During attachment and extending into biofilm maturation, we observed the upregulation of the Pil-Chp surface-sensing complex known to mediate attachment [6], whereas no upregulation of the complementary Wsp system was noted. Notably, transcription of *pilA, pilB, pilD* and *pilGHIJK-chpABCDE,* encoding type IV pili structural and signal transduction proteins, and of *cyaB* and *vfr,* which encode an adenylate cyclase and its associated cyclic-AMP-controlled transcriptional regulator Vfr, was upregulated between 2.00- and 7.37-fold relative to biofilm dispersal.

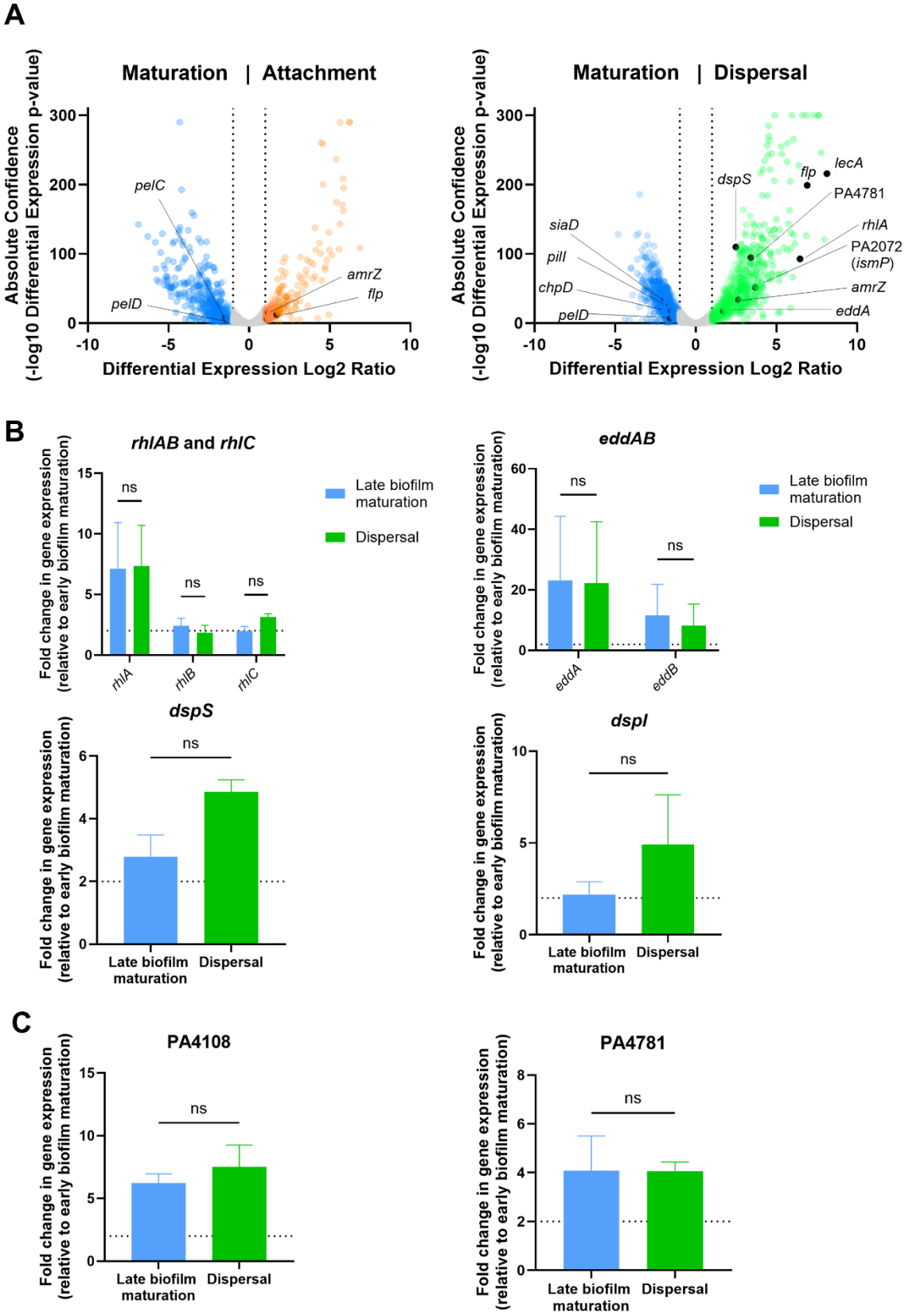
Biofilm maturation involves synthesis pathways for extracellular polysaccharides and eDNA, which are the main components of the biofilm matrix [1]. During biofilm maturation, we observed the upregulation of the exopolysaccharide Pel synthesis operon *pelBCDEF* but not *pelG* relative to attachment (2.22 – 2.26-fold) and dispersal stages (2.16 – 2.99-fold) (Fig 3A, Table S1) [21]. Additionally, the alternative respiration process of denitrification is known to be active during biofilm maturation due to cell layering inside the biofilm restricting oxygen availability [22]. Accordingly, we

found biofilm cells to upregulate the transcription of the nitrite reductase complex (*nirSMCFDLG*) by up to 10-fold relative to attachment.

Planktonic cells at the biofilm dispersal stage, relative to attachment and biofilm maturation stages, showed an increased transcription of genes involved in eDNA hydrolysis (*eddA,* 3.28-fold; and *eddB,* 2.95-fold; but not *endA*) (Fig 3A, Table S1), suggesting the remodelling of the extracellular matrix to allow the escape of planktonic cells. We also observed a pronounced upregulation of quorum-sensing–regulated genes previously shown to drive biofilm dispersal. The *rhlA*, *rhlB*, and *rhlC* genes, which encode enzymes for the biosynthesis of the rhamnolipid biosurfactant implicated in quorum-sensing–mediated dispersal [39–42]were upregulated by 88-, 45- and 12-fold, respectively (Fig 3A, Table S1) [23–26]. Another quorum-sensing pathway activated during dispersal involved the fatty acid signal cis-2-decenoic acid [25,27]. This fatty acid signal is produced and sensed by the proteins encoded by *dspI* and *dspS*, which were upregulated by 2.04- and 5.59-fold, respectively, during dispersal relative to the biofilm maturation stage (Fig 3A, Table S1). Interestingly, genes associated to biofilm maturation were also found upregulated during the dispersal stage. Adhesion factors, such as lectins *lecA* and *lecB* were highly upregulated during dispersal by 279-fold and 115-fold, respectively, relative to the biofilm maturation stage. Similarly, *flp*, which encodes the main subunit of the type IVb pili adhesin was upregulated by 120-fold, together with several alginate biosynthesis genes (*alg8*, 2.26-fold*; algX,* 2.16-fold*; algJ*, 2.39-fold; *algA,* 2.74-fold) and their associated regulators *algU* (2.49-fold)*, algR* (5.76-fold) and *amrZ* (6.27-fold) (Fig 3A, Table S1). These findings were consistent with previous studies showing that dispersed cells had significantly increased functional adhesion relative to planktonic cells [28]. Therefore, our RNA-seq data captured transcriptional changes associated with previously documented attachment, biofilm maturation and dispersal responses, further underscoring the consistency of our dataset with established biofilm regulatory pathways and the robustness of our analytical methods.

To further validate the upregulation of these known dispersal pathways, we extracted RNA from biofilms at 4 h (early maturation), 8 h (late maturation), and planktonic cells collected at 12 h (dispersal) and measured the transcript levels of representative genes involved in rhamnolipid

200 synthesis (*rhlAB, rhlC*), eDNA degradation (*eddA, eddB*), and cis-2-decenoic acid signalling (*dspI,*
201 *dspS*) by RT-qPCR (Fig 3B). Relative to maturing biofilms, all genes were upregulated in mature and
202 dispersed biofilms. These findings further validate our RNA-seq data and indicate that mature
203 biofilms may begin to transcriptionally activate dispersal pathways before the onset of active
204 detachment.


A
Maturation | Attachment
Maturation | Dispersal
Absolute Confidence
(-log10 Differential Expression p-value)
Differential Expression Log2 Ratio
pelC
pelD
amrZ
flp
siaD
pilI
chpD
dspS
lecA
PA4781
rhlA
PA2072
(ismP)
eddA
B
rhlAB and rhlC
eddAB
dspS
dspI
Fold change in gene expression
(relative to early biofilm maturation)
Late biofilm maturation
Dispersal
ns
rhlA
rhlB
rhlC
eddA
eddB
C
PA4108
PA4781

205

206

Figure 3. Genes involved in dispersal mechanisms are upregulated during late biofilm maturation and dispersal compared to early biofilm maturation. (**A)** Comparison of the transcriptomes of planktonic cells during attachment or dispersal to that of sessile cells during biofilm maturation. Genes containing Log2-fold change transcription values >|1| were highlighted in biofilm stage specific colours. (**B and C)** RT-qPCR analysis of canonical dispersal genes (*rhlA, rhlB, rhlC, eddA, eddB, dpsS* and *dspI*) and the PDEs PA4108 and PA4781, which were identified as putatively involved in dispersal. RNA samples were extracted at different time points with Means ± SEM shown.

Specific c-di-GMP enzymes characterise the biofilm maturation and dispersal transcriptomes

Cyclic di-GMP (c-di-GMP) is a key signalling molecule governing the transition from biofilm maturation to dispersal. Considering that the roles of enzymes responsible for its synthesis and breakdown are stage-specific, we also analysed our transcriptomic data to identify genes encoding enzymes with predicted PDE (EAL or HD-GYP) or DGC (GGDEF) domains (Table 1). Expectedly, genes encoding proteins essential for biofilm development (*siaD,* 3.73-fold and *pipA,* 2.08-fold) were upregulated during biofilm maturation [29–32]. Genes implicated in the formation of large microcolonies [30], including PA4396 (2.08-fold), *fimX* (2.77-fold), and PA5442 (2.36-fold) were also significantly upregulated. In contrast, we observed an upregulation of a combination of PDEs and DGCs in cells at the dispersal stage, including sensory transducers of dispersal cues *nbdA* (7.56-fold) and *nicD* (6.73-fold) [33–35]. Additionally, upregulation of *ismP* (12.99-fold) was noted, which encodes an iron sensor that under low iron conditions hijacks and inhibits a cognate DGC ImcA [36].

Table 1: Differentially regulated genes encoding c-di-GMP-modifying enzymes during biofilm maturation and dispersal.

Locus Tag	Gene name	Domain(s)	Activity	Fold-change
Biofilm maturation				(vs Dispersal)
PA0169	*siaD*	GGDEF	DGC	3.73
PA0285	*pipA*	GGDEF-EAL	PDE	2.08
PA4396	-	GGDEF	-	2.08

PA4959	*fimX*	GGDEF-EAL	PDE	2.77
PA5442	-	GGDEF-EAL	PDE	2.36
Biofilm dispersal				(vs Maturation)
PA0290	-	GGDEF	-	2.73
PA0575	*rmcA*	GGDEF-EAL	PDE	3.51
PA0847	-	GGDEF	DGC	2.09
PA0861	*rbdA*	GGDEF-EAL	PDE	3.03
PA1878	-	HD-GYP	-	3.39
PA2072	*ismP*	GGDEF-EAL	-	12.99
PA2572	-	HD-GYP	-	8.22
PA2771	-	GGDEF	DGC	2.75
PA3311	*nbdA*	GGDEF-EAL	PDE	7.56
PA3825	-	EAL	PDE	1.25
PA4108	-	HD-GYP	PDE	5.60
PA4781	-	HD-GYP	PDE	10.70
PA4929	*nicD*	GGDEF	DGC	6.73

Interestingly, dispersed cells upregulated all four genes encoding proteins with HD-GYP phosphodiesterase domains present in the *P. aeruginosa* PAO1 genome (PA1878, PA2572, PA4108 and PA4781) (Table 1). Two of these, PA4108 and PA4781 were previously reported to have PDE activity and to be necessary for swarming and the production of virulence factors [37,38]. We further validated the transcriptional changes of these two genes using RT-qPCR (Figure 3C), confirming an increase in expression of 7.5-fold and 4-fold, respectively, during dispersal relative to maturing biofilms at 4 h. Moreover, the transcription of both genes was also upregulated in mature biofilms (6.2- and 4-fold; Figure 3C), indicating a similar pattern of transcriptional priming prior to dispersal. Altogether, our results indicate that canonical stage-specific c-di-GMP modulation responses are accurately reflected in our transcriptomic dataset and suggest a potential undescribed role of HD-GYP-containing PDEs in mediating biofilm dispersal.

Transcriptional biomarkers identify the onset of biofilm dispersal

244 Since the obtained transcriptomic profiles recapitulated canonical pathways associated with
245 attachment, maturation, and dispersal, our datasets are uniquely well suited to identify genes
246 associated with the dispersal phase of *P. aeruginosa* to serve as biomarkers of biofilm dispersal. We
247 reasoned that genes directly involved in biofilm dispersal would exhibit inverse regulation between
248 the biofilm maturation and dispersal stages relative to the initial attachment stage. Thus, pro-dispersal
249 genes would be repressed during maturation but strongly upregulated during dispersal, or vice versa.
250 To identify candidate biomarkers, we applied these selection criteria and identified 14 upregulated and
251 8 downregulated genes that could potentially serve as dispersal biomarkers (Table 2).

252 Among the upregulated genes, *cheR2* (2.1-fold, Table 2) encodes a methyltransferase component of
253 the Che2 chemotaxis pathway, which interacts with the heme-containing oxygen sensor McbP/Aer2
254 and the coupling protein CheW2 to modulate virulence through a cryptic phosphorylation cascade
255 [39,40]. Another notable gene, *pqqA* (2.55-fold; Table 2), is part of the *pqqABCDE* operon responsible for
256 the biosynthesis of pyrroloquinoline quinone (PQQ), a redox cofactor for the quinoprotein ethanol
257 dehydrogenases ExaA and ExaBC [41]. In addition, several genes under the transcriptional control of the
258 two-component system PprAB were also upregulated. These include *tadA*, *rcpA*, *rcpC*, and *flp* (13,66-
259 , 12.47-, 13.83- and 34.54-fold; Table 2) which are part of a contiguous cluster spanning PA4297
260 (*tadG*) to PA4306 (*flp*). This locus encodes the machinery for the synthesis and secretion of Flp type
261 IVb pili [42]. Notably, two of the dispersal biomarkers *amrZ* and PA2588 (*cdpR*) have known roles in
262 modulating biofilm physiology. *amrZ* encodes a recognised transcriptional regulator of dispersal and
263 *cdpR* is a quorum sensing and virulence transcriptional regulator [43–45].

264 Several uncharacterised genes, including PA0111, PA0743 and PA1353 were also identified as
265 upregulated biomarkers of biofilm dispersal, many of which appear to be associated with redox
266 processes. While no experimental data are available for PA0111, bioinformatic predictions suggest
267 that the directly upstream gene PA0110 encodes a protein putatively related to a mitochondrial
268 cytochrome *c* oxidase. Additionally, PA0112 and PA0113 are predicted to encode a heme A synthase
269 and a protoheme IX farnesyltransferase, respectively, implicating these loci in cytochrome maturation
270 or electron transport. Similarly, PA0743, which encodes an L-serine dehydrogenase, was also

upregulated, potentially reflecting altered amino acid catabolism related to redox homeostasis during biofilm dispersal. No functional predictions are currently available for PA1353, which was also part of the identified dispersal biomarkers.

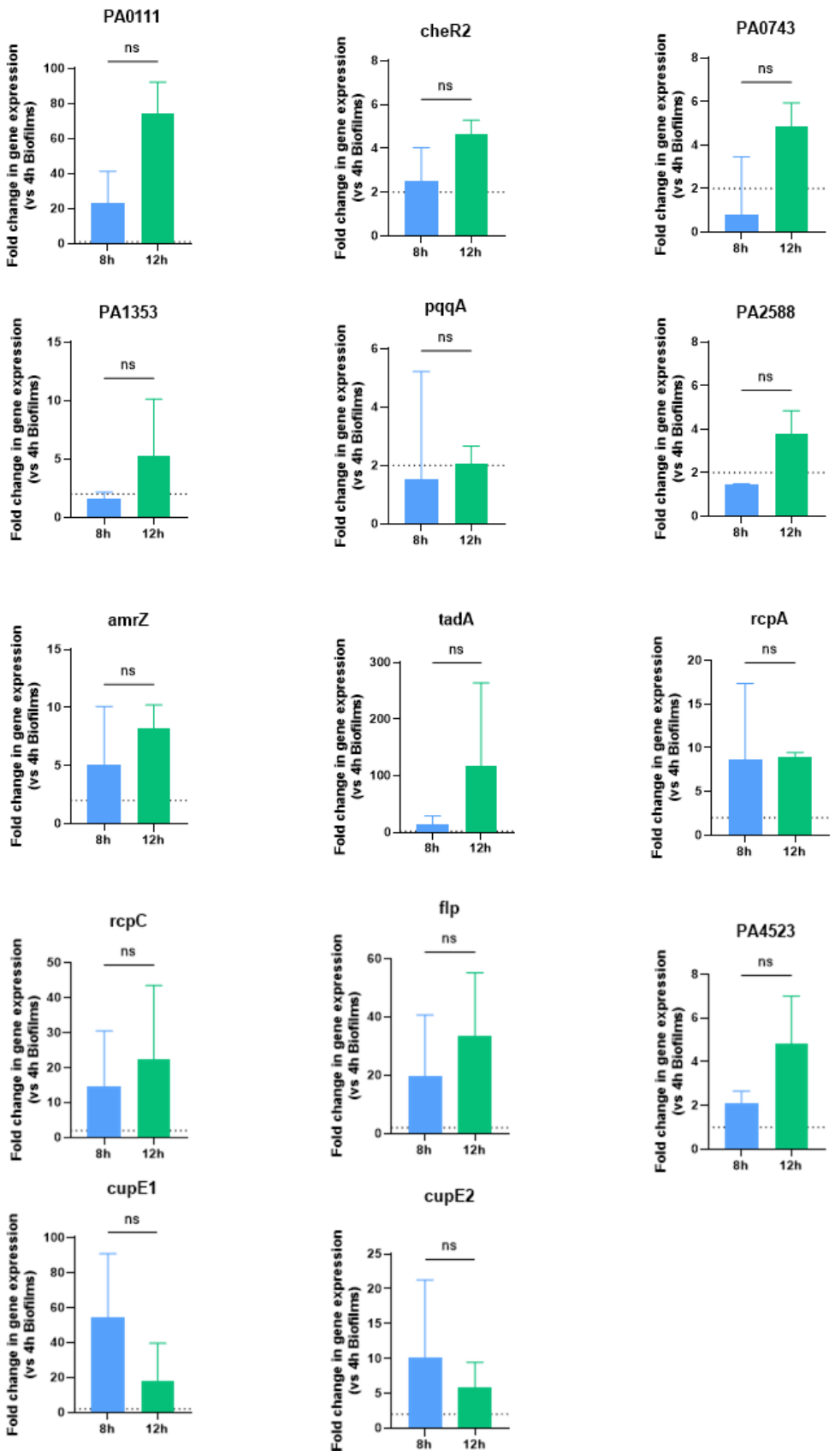
Table 2. Genes differentially regulated at the biofilm maturation and dispersal stage compared to the attachment stage.

Gene	**Fold change in Biofilm maturation [a]**	**Fold change in Biofilm dispersal [a]**	**Gene product**
Downregulated at 4h and upregulated at 8h			
PA0111	-2.01	12.21	hypothetical protein
*cheR*2	-2.22	2.10	chemotaxis protein methyltransferase
PA0743	-2.58	3.12	NAD-dependent L-serine dehydrogenase
PA1353	-2.36	3.32	hypothetical protein
pqqA	-5.10	2.55	coenzyme PQQ synthesis protein A
PA2588	-2.03	3.01	transcriptional regulator
amrZ	-2.30	2.55	alginate and motility regulator Z
tadA	-2.17	13.64	ATPase TadA
rcpA	-2.13	12.47	type II/III secretion system protein
rcpC	-2.07	13.83	hypothetical protein
flp	-3.27	34.54	type IVb pilin Flp
PA4523	-2.31	3.41	hypothetical protein
cupE1	-2.11	9.58	fimbrial subunit CupE1
cupE2	-2.04	3.95	fimbrial subunit CupE2
Upregulated at 4h and downregulated at 8h			
PA0485	2.93	- 2.91	hypothetical protein
PA1170	2.43	-4.76	hypothetical protein
metH	2.45	-2.13	B12-dependent methionine synthase
ilvH	3.53	-2.35	acetolactate synthase small subunit
ilvI	4.69	-2.57	acetolactate synthase 3 catalytic subunit
cysN	2.1	-2.62	bifunctional sulfate adenylyltransferase subunit1/adenylylsulfate kinase
PA4889	2.06	-14.92	oxidoreductase

PA4907	2.41	-2.27	short-chain dehydrogenase

[a] Relative to samples collected during attachment.

We restricted our biomarker screen to upregulated transcripts, as elevated expression levels are more consistently detected and validated across analytic platforms. Due to the similarities in transcriptomic profiles between planktonic and biofilm cells collected during dispersal, we hypothesised that biofilms become primed for dispersal prior to phenotypically manifesting the response. Therefore, we collected samples from mature biofilms at 8 h and from dispersed cells at 12 h and validated these biomarker genes by RT-qPCR (Fig 4). Our results confirmed the upregulation of all fourteen genes during dispersal, with four of these (PA0743, PA1353, *pqqA*, and PA2588) only being significantly upregulated at 12 h (Fig 4). Genes PA0111, *cheR2*, *tadA*, *rcpC*, *flp*, and PA4523 showed a pattern where transcript abundance increased in mature biofilms (2.5- to 23-fold) and peaked during dispersal (4.6- to 117-fold relative to early biofilms) (Fig 4). In contrast, the transcription of *cupE1* and *cupE2* fimbrial genes peaked in mature biofilms (28.6- and 10.1-fold) but sharply declined upon dispersal (6.1- and 5.9-fold) (Fig 4). This transient transcriptional profile suggests that CupE fimbriae genes are likely characteristic of mature biofilms. In contrast, transcription of PA0111, *cheR2, tadA, rcpC, flp* and PA4523 peaked at dispersal, indicating that matured biofilms become primed for dispersal prior to its onset (Fig 4). Altogether, these biofilm dispersal markers exhibit strong reproducibility, with 12 out of 14 becoming progressively upregulated during the transition from mature biofilms to dispersal.



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296 **Figure 4. Validation of the transcriptional biomarkers of biofilm dispersal.** Differences in transcriptional regulation

297 measured by RT-qPCR for the indicated genes from total RNA samples extracted at 4 h (early biofilm maturation), 8 h (late

biofilm maturation) and 12 h (dispersal). Fold-changes at 8 h and 12 h are relative to 4 h. Means ± SEM are shown. Two technical replicates are represented.

Discussion

The study of transcriptional changes between each biofilm stage provides valuable insights into the key cellular signalling pathways underlying their transition between sessile and motile lifestyles. Here, we characterised the transcriptional profiles of cells undergoing attachment, biofilm maturation and dispersal, and identified 23 biomarkers characterising the dispersal response, 14 of which comprised a subset strongly upregulated upon the initiation of dispersal.

Cells undergoing attachment sense and respond to surface contacts through mechanosensory systems such as the Pil-Chp machinery, which relies on type IV pili-mediated twitching motility [46,47]. In our study, we report an upregulation of type IV pili biogenesis genes (*pilA, pilB, pilD*) and of the mechanosensory system Pil-Chp (*pilGHIJK-chpABCDE*) during the attachment and biofilm maturation stages compared to dispersing cells. These were accompanied by the upregulation of *fimX*, which encodes a putative phosphodiesterase essential for the assembly of type IV pili, and of *cupA1* encoding the main subunit of the CupA adhesin [48]. Our findings therefore align with previous studies indicating that type IV pili are indispensable to surface-attachment in microplates [49], and are consistent with prior reports that surface sensing promotes the transcriptional upregulation of genes involved in type IV pilus biogenesis [50].

The intracellular messenger c-di-GMP acts as a master regulator of the bacterial lifestyle, with its accumulation leading to biofilm formation [8]. Our dataset revealed the upregulation of several stage-specific genes encoding proteins with validated or hypothetical c-di-GMP modulating activity. Specifically, *siaD* was the most upregulated during biofilm maturation, which encodes a DGC involved in early microcolony formation and adhesion [46,51]. In contrast, biofilm dispersal is associated with increased PDE activity and decreasing intracellular c-di-GMP [52]. This was reflected in our dataset, where 6 genes encoding functionally active PDEs were upregulated. Genes *rbdA* and *nbdA*

324 encode enzymes with well-described roles in biofilm dispersal. Both have been reported to modulate
325 flagellar motility, with RbdA being a key component of the BdlA-regulated core dispersal response
326 [53,54]. Moreover, four upregulated genes (PA1878, PA2572, PA4108 and PA4781) encode PDEs
327 carrying the HD-GYP motif [55]. While the role of these genes in the biofilm dispersal stage is unclear,
328 it has been reported that disruption of PA4108 and PA4781 resulted in higher intracellular c-di-GMP
329 and decreased swarming activity, whereas PA2572 modulated swarming motility [55,56]. We here
330 validated by RNA-seq and RT-qPCR that both PA4108 and PA4781 are strongly upregulated during
331 late biofilm maturation and dispersal, which suggests a potential role in initiating dispersal responses.
332 Additionally, recent reports showed that deletion of *cdpR* led to increased biofilm biomass and
333 decreased swarming motility [45]. Considering that the upregulation of *cdpR* is strongly controlled by
334 the quorum sensing regulator RsaL [44], the stage-specific transcriptional activation of *cdpR* would
335 therefore suggest that dispersal in closed systems is mediated by a global quorum sensing-mediated
336 swarming response.

337 The synthesis of the rhamnolipid biosurfactant is important to the quorum sensing-mediated swarming
338 motility and dispersal [24]. In closed culture systems, rhamnolipid was proposed to drive biofilm
339 dispersal [57]. Here we report that the rhamnolipid and swarming modulator PA2572 together with
340 several rhamnolipid-associated genes were highly upregulated during dispersal. Moreover, four of the
341 identified PDEs upregulated during dispersal, including PA4108, PA4781, *nbdA* and *rbdA,* were
342 previously shown to influence the synthesis of rhamnolipid leading to loss of biofilm biomass [19,34,56].
343 Altogether, these data suggest biofilm dispersal in closed systems may be driven by rhamnolipids.

344 Among the upregulated biomarkers, *amrZ* was reported as a global regulator controlling the
345 upregulation of genes related to alginate biosynthesis and Type IV pili twitching motility while
346 downregulating Pel synthesis, therefore driving biofilm dispersal [43,58]. In contrast, *tadA*, *rcpA*, *rcpC*,
347 *flp, cupE1* and *cupE2* were previously reported to be responsible for cell adhesion which are regulated
348 under the PprAB two-component system [59,60]. Our data therefore support previous studies indicating
349 that dispersing cells exhibit a hyperadhesive potential that primes them to re-attach to surfaces [28].
350 Additionally, we identified PA0110, *cheR2*, *pqqA,* PA0743, PA1353 and PA4523 to be uniquely

351 associated to the dispersal stage, yet their specific roles remain to be defined. Their marked
352 upregulation during dispersal warrants further investigation.

353 Understanding biofilm dispersal could prove a critical step towards combating chronic, biofilm-
354 associated infections. As dispersing cells become resensitised to antimicrobial treatment once they are
355 released from the protective biofilm environment, the underlying transcriptional changes occurring
356 during biofilm dispersal hold promise to unveiling the next generation of antibiofilm treatments and
357 adjuvants. We here report the transcriptional profiles of the *P. aeruginosa* biofilm life cycle, including
358 attachment, biofilm maturation and dispersal stages in closed systems, which can be used as
359 benchmarks for future studies. Additionally, we propose a robust set of biofilm dispersal biomarkers
360 with the potential to be used in the high throughput screens supporting the discovery and development
361 of novel dispersal-inducing antibiofilm therapeutics.

362 Materials and Methods

363 *Strains, media and culture conditions*

364 *Pseudomonas aeruginosa* PAO1 wild-type (WT). Cultures were routinely grown overnight in LB
365 (lysogeny broth) media at 37°C, 200rpm before incubation in fresh M9 media (9 mM NaCl, 22 mM
366 KH_2PO_4, 48 mM Na_2HPO_4, 19 mM NH_4Cl and 2 mM $MgSO_4$, 100 µM $CaCl_2$, 0.4% glucose, pH 7.0)
367 for each experiment. Where appropriate, cultures were supplemented with 10µg/ml of gentamicin.

368 *Biofilm kinetics and microscopy*

369 Biofilms were grown on Nunc 24-well plates (ThermoFisher Scientific) or tissue culture flasks (Nunc,
370 ThermoFisher Scientific) by inoculating 10^7 colony forming units (CFU)/ml of overnight bacterial
371 cultures for 2 h, 4 h, 8 h and 12h in 1 ml of M9 media at 37 °C and 180 rpm (Fig S2). At the indicated
372 time points, supernatants were removed, and wells were delicately washed with 1ml phosphate
373 buffered saline (PBS, Gibco) to remove planktonic cells. Microscopy (400x; PrimoVert, Zeiss) was
374 used to confirm the removal of planktonic cells and preservation of biofilm structures. Remaining
375 biofilm cells were stained with 0.1% (w/v) crystal violet dissolved in 6.25% (v/v) methanol. Stained

376 biofilms were washed twice with PBS and solubilised with ethanol. Biofilm biomass was quantified
377 by measuring the optical density at 550 nm (OD_{550}) using a SPECTROStar Nano microplate reader
378 (BMG LabTech). Micrographs of stained biofilms were taken via microscopy as described above.

379 *RNA sequencing and analysis*

380 Biofilms were cultured and dispersed as previously described with some adjustments [18]. Briefly, tissue
381 culture flasks (Nunc, ThermoFisher Scientific) were inoculated with 10^7 CFU/ml cells in 50ml of M9
382 medium from overnight cultures. Cultures were incubated at 37°C shaking at 70rpm for 2, 4 or 8h. At
383 2 h and 8 h, planktonic cells (10^8) were collected from the liquid phase and immediately mixed with 2
384 volumes of RNA-protect solution (QIAGEN). Biofilm cells were gently rinsed with 10 ml of PBS and
385 resuspended in 6 ml of RNAprotect mixed with 3ml of PBS using cell scrapers. Cells (10^8) were
386 subsequently pelleted at 5000 g (10 min, 25 °C). RNA was extracted with the RNeasy mini kit
387 (QIAGEN, Cat# 74104) as per the manufacturer's protocol for Gram negative bacteria grown in
388 minimal media. Extracted RNA samples were treated with DNAse I and rRNA was removed using
389 streptavidin-coated magnetic beads (BGI Genomics, Shenzhen, China). Library was prepared by RNA
390 fragmentation and cDNA synthesis followed by the ligation of adaptors at the 3' end of cDNA
391 fragments. These were further amplified by PCR and single-stranded fragments were circularised for
392 downstream DNBSEQ PE100 sequencing (BGI Genomics, Shenzhen, China). Clean reads were
393 mapped to the *P. aeruginosa* PAO1 reference genome (GenBank Accession number AE004091.2)
394 with Bowtie2 and comparison of expression levels between sample groups (attachment, biofilm
395 maturation and dispersal) was done with DESeq2 in Geneious Prime v2024.0.7.

396 The PCA plot was generated by extracting the reads per kilobase of transcript per million mapped
397 reads (RPKM) of every gene at each one of the stages (Table S1). Then, genes with <|2-fold|
398 transcription or *P*-value >0.01 for all groups were discarded, and the remaining differentially
399 regulated genes were plotted using GraphPad Prism 10.4.1 (Graph Pad, USA).

400 *qRT-PCR*

401 To validate RNA-seq data, total RNA was extracted from cells harvested from different biofilm stages
402 as described above. cDNA was transcribed using 1 µg of total RNA using the SuperScript III first-
403 strand synthesis kit (Invitrogen) as per the manufacturer's protocol. cDNA samples were then used as
404 templates for RT-qPCR conducted with the Quantinova SYBR Green master mix (Qiagen) as per the
405 manufacturer's protocol. The housekeeping gene *recA* was used as a control. Each sample was
406 independently assayed twice with the gene-specific primers listed in Table S2. Relative gene
407 expression was calculated using the $2^{-\Delta\Delta CT}$ method [61]. Transcriptional fold changes were plotted using
408 GraphPad Prism.

409 Acknowledgements

410 This work was funded by an Australian Research Council project grant (DP210101317), the Max
411 Planck Queensland Centre on the Materials Science of Extracellular Matrices, and a QUT Amplify
412 Scholarship provided by the Queensland University of Technology (Australia) to XB. The Ian Potter
413 Foundation sponsored the CLARIOStar high-performance microplate reader (BMG, Australia). The
414 funders had no role in study design, data collection and analysis, decision to publish, or preparation of
415 the manuscript. The authors would like to thank Professor Robert EW Hancock (University of
416 Columbia) for providing the *P. aeruginosa* PAO1 strain used in this study.

417 Competing interests

418 MT is an employee of the GSK group of companies. All other authors declare no competing interests.
419 This research was conducted in the absence of any commercial or financial relationships that could be
420 constructed as a potential conflict of interest.

421 Author contributions

422 JQ and MT conceptualised the project. XB, JQ and MT contributed to experimental design. XB
423 conducted all experiments, and contributed to data collection, analysis and visualisation. XB, JQ and
424 MT contributed to data interpretation. JQ, MT and KFS supervised the project. KFS and MT obtained

the funding. XB wrote the manuscript draft, JQ substantially revised the manuscript, all authors edited the manuscript.

Data availability

The RNA-Seq data have been deposited in the NCBI Short Read Archive (SRA) database with accession code PRJNA1291829.

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