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***In vitro* and *in vivo* generation and characterization of *Pseudomonas aeruginosa* biofilm
dispersed cells via c-di-GMP manipulation**

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Abstract

C-di-GMP is a global secondary bacterial messenger that controls the formation of drug resistant multicellular biofilms. Lowering the intracellular c-di-GMP content can disperse biofilms and is proposed as a biofilm eradication strategy. However, freshly dispersed biofilm cells exhibit a physiology distinct from biofilm and planktonic cells, and might play a clinically relevant role in infections. Here, we present *in vitro* and *in vivo* protocols for generating and characterizing dispersed cells from *Pseudomonas aeruginosa* biofilms by reducing the intracellular c-di-GMP content through modulation of phosphodiesterases (PDEs). Unlike conventional protocols that demonstrate biofilm dispersal by biomass quantification, our protocols enable physiological characterization of the dispersed cells. Biomarkers of dispersed cells are identified and quantified, which serve as potential targets for treating the dispersed cells. The *in vitro* protocol can be completed within 4 d, while the *in vivo* protocol requires 7 d.

Keywords: Biofilm dispersal, *Pseudomonas aeruginosa*, c-di-GMP, dispersed cells

EDITORIAL SUMMARY: This protocol generates and characterizes dispersed cells from *Pseudomonas aeruginosa* biofilms *in vitro* and *in vivo* by reducing the intracellular c-di-GMP content. Dispersed biofilm cells play an important role in transmission of infections.

TWEET: Generating and characterizing *P. aeruginosa* #biofilm dispersed cells via c-di-GMP manipulation #antibiotic_resistance

1 **Introduction**

2 Biofilm formation is now considered the dominant mode of growth for bacteria in natural
3 habitats, clinical environments and diseases ¹⁻³. Multiple mechanisms are involved in the
4 transition from the planktonic to the biofilm mode of growth by bacteria, including attachment of
5 cells to a surface, growth and aggregation into microcolonies, and secretion of extracellular
6 polymeric substances (EPS) to form mature biofilm structures ⁴⁻⁶. Dispersal of cells from the
7 biofilm to the environment concludes the growth cycle ^{1,7}. While biofilm formation confers
8 bacteria the ability to sustain life in harsh and hostile environments, the process of dispersal
9 enables sessile bacteria to detach from the biofilms and relocate to new, vacant niches for
10 colonization ⁸. Biofilm dispersal is particularly important for pathogenic bacteria during
11 transmission from the environment to the host, transmission between hosts and spread of
12 infection within the host ³.

13 The opportunistic pathogen *Pseudomonas aeruginosa* plays an important role in nosocomial
14 infections ⁹. The lung infection of cystic fibrosis (CF) patients caused by biofilm-forming *P.*
15 *aeruginosa* is a clinically important example, which often results in a progressive decline in lung
16 function and eventual mortality ¹⁰⁻¹². Although biofilm dispersal is evidently important in the
17 transmission of diseases ¹³, studies of the roles of dispersal in bacterial pathogenesis are limited.
18 Biofilm dispersal can be induced either by environmental factors ^{14,15} or by genetic programming
19 ¹⁶. The majority of studies dealing with biofilm dispersal have focused on identifying
20 environmental factors (both physical and chemical) that induce dispersal. Physical factors such
21 as shear forces ¹⁷, nutrient depletion ¹⁸, and exposure to chemicals such as glutamate ¹⁵ and nitric
22 oxide ¹⁴ can cause biofilm dispersal.

Bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) was recently identified as a global secondary bacterial messenger that regulates the transition between biofilm (sessile) and planktonic (free swimming) modes of growth¹⁹. The immediate intracellular content of c-di-GMP is determined by the activities of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), which catalyze the formation and degradation of c-di-GMP, respectively¹⁹. Increased intracellular content of c-di-GMP induces biofilm formation by enhancing surface adherence of bacterial cells, cell aggregation and secretion of EPS²⁰. In contrast, lowering intracellular content of c-di-GMP results in biofilm dispersal and enhancement of bacterial motility²⁰. Freshly dispersed cells contain lower c-di-GMP content than planktonic cells and biofilm cells^{16,21}, and show distinct physiology from planktonic and biofilm cells with respect to the susceptibility to antimicrobial agents and iron stress, and virulence against eukaryotic hosts^{16,21,22}.

Protocol development

Novel approaches to controlling biofilm infections, such as dispersing biofilms to render pathogens susceptible, are becoming increasingly necessary given the widespread difficulty in treating biofilm infections by conventional antibiotics. However, biofilm dispersal is one of the least understood stages of the bacterial biofilm life cycle. Chemical stimuli of biofilm dispersal, such as nitric oxide (NO) and glutamate, have been identified^{14,15}, and tested in a variety of biofilm experiments, such as flow cell (continuous-culture) biofilm experiments, 96-well batch cultures and petri dish batch culture assays²³. Low and non-toxic levels of NO are able to regulate the c-di-GMP signaling pathway and induce biofilm dispersal²⁴. NO can disperse biofilms formed by a range of bacterial species, such as *P. aeruginosa*, *Escherichia coli*, *Vibrio cholerae*, *Staphylococcus aureus*, *Bacillus licheniformis*²³ and *Legionella pneumophila*²⁵. In *P.*

1 *aeruginosa*, a biofilm model organism, the chemotaxis transducer BdlA participates in the
2 sensing of NO and activation of the DipA PDE, resulting in lowering intracellular c-di-GMP
3 content and biofilm dispersal^{24,26}. Given the universal and regulatory role of c-di-GMP signaling
4 in microbial biofilms, reducing c-di-GMP content by chemical biology approaches to promote
5 biofilm dispersal, was proposed as a promising biofilm eradication strategy.

6 In a recent proof-of-concept study, decreased intracellular c-di-GMP content induced biofilm
7 dispersal from implants inserted intra-peritoneally in mice²⁷. However, this raises doubts on the
8 safety of liberating large amounts of dispersed bacterial cells into the bloodstream. Hence, it is
9 necessary to examine the dispersed cells to investigate their physiology and role in infections.

10 Current studies of biofilm dispersal include identification of PDEs required for c-di-GMP
11 degradation and biofilm dispersal^{26,28} and chemical or physical stimuli that initiate biofilm
12 dispersal^{14,15,29}. There are two methods currently used to induce c-di-GMP-mediated biofilm
13 dispersal, namely chemical and enzymatic dispersal. For chemical dispersal of biofilms, the
14 chemical stimuli, such as NO-generating sodium nitroprusside (SNP) is added directly to the
15 biofilm. A recent protocol detailed procedures for dispersing *P. aeruginosa* biofilms by using a
16 NO donor³⁰. This protocol can serve as a reference for screening novel biofilm-dispersing active
17 compounds. However, chemical dispersal of biofilms can be unspecific and result in certain
18 pleiotropic effects other than biofilm dispersal. The dispersed cells generated by different
19 chemicals might have different physiology. In contrast, our biofilm dispersal protocols are based
20 on enzymatic degradation of intracellular c-di-GMP content by using the arabinose-inducible
21 YhjH PDE, cloned into the pJN105 plasmid²¹. Hence, our protocols will be more appropriate for
22 studying the specific physiology of dispersed cells. The inducible p_{BAD}-*yhjH* plasmid can also be
23 used in most other Gram-negative bacteria, but is not applicable for bacterial species that do not

possess the c-di-GMP signaling system. Using this system we created methods for generating and characterizing freshly dispersed cells *in vitro* and *in vivo*, which extends previous work in the emerging field of biofilm dispersal that has shown dispersed cells to be physiologically unique compared to both biofilm and planktonic cells^{16,21}.

Applications of c-di-GMP-mediated biofilm dispersal

The protocols described here will be useful for biochemists and clinicians to discover potential drugs targeting biofilm dispersal or dispersed cells, and will allow microbiologists to find new genes involved in the spread of diseases by dispersed cells throughout the host organism.

Currently, there is a great need to better understand biofilm dispersal for improving treatment of biofilm infections. Experiments to investigate biofilm dispersal can be applied for various purposes, as detailed below:

[1] To explore new ways of biofilm dispersal in treatment of biofilm-associated chronic infections. Anti-biofilm compounds that interfere with c-di-GMP signaling and result in biofilm dispersal can be identified from the screening of compound libraries. This allows the potential use of dispersal agents clinically, such as in treating lung infections in cystic fibrosis patients. Antibiotics can also be used synergistically with dispersal agents to kill the dispersed biofilms.

[2] To further characterize the properties of biofilm-dispersed cells, in other bacterial species. Given c-di-GMP signaling is prevalent in most Gram-negative bacteria, biofilm dispersal can be tested in various pathogenic bacteria, such as *Vibrio cholerae*. By studying the physiologies of the dispersed cells from different species, we are able to compare and identify the universal traits of dispersed cells, such as enhanced virulence levels. Different chemical stimuli can also be

tested in biofilm dispersal, to determine whether or not their working mechanisms require c-di-GMP signaling.

[3] To identify potential drug targets of dispersed cells and establish ways to eliminate them, thus preventing the spread of infections. As the role of dispersed cells is to colonize new areas, understanding dispersed cells may provide important information to halt the spread of pathogenic bacteria, either within the host; from host to host or from the environment to the host. Given the unique physiology of the dispersed cells, the drugs used to eradicate them should be slightly different than those for treating planktonic or biofilm cells. The eradication of dispersed cells will prevent the dissemination of infections.

[4] To discover biomarkers unique to dispersed cells, for possible diagnosis of infection spread. Identified biomarkers can potentially be used to establish the presence of dispersed cells in the host and to diagnose the spread of infection throughout the host.

The applications of our protocols are not limited to clinical settings, but extend to use in environmental and engineering settings. The biofouling of membrane reactors for wastewater treatment and water distribution pipelines is largely attributed to biofilm formation³¹. Biofilms also pose a great contamination threat in food industries, often resulting in food spoilage and breaches in food safety³². The application of biofilm dispersing compounds in these settings can increase the lifespan of the industrial facilities³³, but also generates potentially virulent dispersed cells. It is therefore of interest to characterize biofilm dispersed cells from these environmental and engineering settings.

Our protocol enables dispersed cells to be generated from biofilms via c-di-GMP manipulation, thus allowing the unique genetic and phenotypic characteristics of dispersed cells to be determined with reproducibility. The identification of biomarkers will help in establishing the

spread of biofilm-mediated infections. While biofilm dispersal is a useful and potentially appealing method of treating biofilm-mediated chronic infections, the risk of releasing bacteria into the bloodstream and causing sepsis has called for safer biofilm dispersal protocols involving killing of dispersed cells. Hence, it is also clinically important to study the tolerance of *in vivo* models to biofilm dispersal and discover potential treatment methods that eliminate dispersed cells.

Limitations of protocols for biofilm dispersal

Only limited numbers of microbial species have currently been studied to characterize biofilm dispersal including the Gram negative bacteria, *Escherichia coli*, *P. aeruginosa*, *Pseudomonas putida* and *Salmonella enterica*, and Gram positive *Streptococcus pneumoniae*, *Listeria monocytogenes* and *Mycobacterium tuberculosis*, and the fungal pathogen *Candida albicans*³⁴⁻⁴⁰.

Given that most bacteria can form and disperse biofilms, our protocols could be applied to diverse bacterial species and expanded to study more complex multi-species bacteria models.

The protocols described here are limited to research and clinical settings, but they can potentially be expanded to study environmental and engineering-based biofilms such as those involved in surface biofouling and toxic chemical bioremediation.

Our protocol is limited to c-di-GMP regulated biofilm dispersal and is not applicable to biofilms formed by bacterial species that do not possess the c-di-GMP signaling systems (such as most of the Gram-positive species). Moreover, our protocol can only be used for detailed characterization of the physiology of *in vitro* dispersed cells, as *in vivo* dispersed cells rapidly change their physiology once exposed to the host's immune cells.

1

2 **Experimental Design**

3 The main steps of *in vitro* and *in vivo* biofilm dispersal are shown sequentially below:

4 *In vitro* biofilm dispersal (Fig. 1), growing biofilms (Steps 1-4): *Pseudomonas aeruginosa*

5 biofilms are grown *in vitro* (such as in microtitre plates and flow chambers). The *in vitro* assay

6 using microtitre plates is simple to construct and suitable for high throughput screening of novel

7 dispersal regulating genes or dispersal agents.

8 *In vitro* biofilm dispersal (Fig. 1), biofilm dispersal (Steps 5-7): Biofilm dispersal can be

9 achieved through genetic approaches. Biofilms of the *P. aeruginosa* PAO1 strain carrying the

10 p_{BAD}-yhjH plasmid²⁷, which contains an _L-arabinose inducible yhjH PDE gene, are cultivated

11 and when treated with _L-arabinose, express yhjH, which results in biofilm dispersal²¹.

12 *In vitro* biofilm dispersal (Fig. 1), quantification of dispersed cells and biofilms (Steps 8-18):

13 Quantification of dispersed cells and remaining biofilms is required to ensure that biofilm

14 dispersal is activated. Dispersed cells can be quantified by measuring colony forming units (CFU

15 ml⁻¹) or optical density (OD₆₀₀). Biofilms can be quantified using crystal violet staining. To

16 verify the efficacy of our protocols, we will mention various forms of quality control to be

17 conducted.

18 *In vitro* biofilm dispersal (Fig. 1), identification of biomarkers unique to *P. aeruginosa* dispersed

19 cells: Biomarkers are important in identifying dispersed cells. As dispersed cells possess low

20 intracellular content of c-di-GMP, c-di-GMP is the universal biomarker for dispersed cells and

21 can be directly quantified by high-performance liquid chromatography (HPLC, Steps 19-35). As

22 transcriptome data and phenotypes of the *P. aeruginosa* dispersed cells were published¹⁶, we can

use both transcriptional fusion based biosensors and dispersed cell-specific phenotypes as biomarkers of dispersed cells. The transcriptional fusion biosensors used here are p_{cdrA} -gfp⁴¹ and p_{pvdA} -gfp⁴². The CdrA is an adhesion protein in biofilms produced by *P. aeruginosa* under high c-di-GMP levels⁴¹. The p_{cdrA} -gfp expresses the unstable GFP version (ASV), thus it is useful in real-time to determine the transition from biofilm to planktonic phase⁴¹ (Steps 36-38). Next, the *pvdA* encodes an enzyme that is involved in the pyoverdine synthesis pathway and is upregulated by high c-di-GMP levels in biofilm formation^{43,44} (Steps 39-41). Both of these two transcriptional fusions were recently shown to be down-regulated in dispersed cells^{16,21}. The phenotypic assay is based on the measurement of pyoverdine (Steps 42-44) - a siderophore produced by *P. aeruginosa* biofilms to sequester iron from the surroundings⁴⁵. It is produced by the *pvd* operon and upregulated by high c-di-GMP levels^{44,46}. However, dispersed cells produce lower levels of pyoverdine than planktonic and biofilm cells¹⁶. Hence, these phenotypes could be used as biomarkers for evaluating c-di-GMP mediated dispersed cells.

In vivo biofilm dispersal (Fig. 2). The mouse implant biofilm model is clinically relevant as it enables the testing of antimicrobial compounds in physiologically relevant environments, allowing investigation of host-pathogen interactions. However, the implant model would have to be adjusted slightly (such as the size of the implant materials) accordingly in the study of infections with other bacterial species.

In vivo biofilm dispersal (Fig. 2), biofilm growth on implants (Steps 45-67): *P. aeruginosa* PAO1/ p_{BAD} -yjhH biofilms are firstly grown on an implant and inserted intraperitoneally into the mouse abdomen. The versatility of the assay allows the biofilms to be grown on many chronic infection models such as catheters in a urinary tract infection model or microbeads in a lung infection model.

1 *In vivo* biofilm dispersal (Fig. 2), dispersal of biofilm *in vivo* (Step 68): After the mice are
2 challenged with the biofilm implant for 24 hours, 2% w/v L-arabinose is injected
3 intraperitoneally to the site of the implant to induce the expression of the *yhjH* PDE thereby
4 initiating biofilm dispersal.

5 *In vivo* biofilm dispersal (Fig. 2), quantification of dispersed cells and biofilms (Steps 69-80): To
6 quantify the extent of induced dispersal in the mouse tissue, CFU ml⁻¹ of dispersed cells are
7 measured in two locations: in the intraperitoneal cavity where the implant resides and in the
8 spleen²⁷. During biofilm dispersal, the cells on the implant enter into the surrounding sterile
9 intraperitoneal cavity, which is thus a prime location for quantifying the CFU ml⁻¹ of dispersed
10 cells. As dispersed cells can also enter the bloodstream, the CFU ml⁻¹ of dispersed cells is also
11 quantified in the spleen to observe the spread of infection. The remaining biofilm on the implant
12 after dispersal is also quantified by CFU ml⁻¹ to check if the biofilm load has been alleviated.
13 Biomarkers described for the *in vitro* assays can also be utilized similarly for *in vivo* models.

14 15 **Controls.**

16 *In vitro* biofilm dispersal. As a negative control, the *P. aeruginosa* PAO1/p_{BAD}-*yhjH* biofilms are
17 treated with ABTGC without L-arabinose. This will prevent the activation of the *yhjH* PDE and
18 avoid complete biofilm dispersal. However, there will still be basal but very low levels of
19 biofilm dispersal, which is physiologically relevant for any bacterial biofilm.

20 Next, to identify the characteristics unique to dispersed cells in contrast to planktonic cells, both
21 planktonic and dispersed cells should be grown under the same conditions for comparison of
22 their biomarkers.

1 Lastly, the time of collecting and assessing dispersed cells is crucial. The time required for
2 biofilm dispersal might vary depending on the bacterial species and efficiency of the dispersal
3 agents. Moreover, the time required for transition from dispersed cells to planktonic cells might
4 vary depending on the bacterial species and culture conditions. While observation of the
5 transition from biofilm to dispersed cells is apparent when bacterial cells move from one location
6 to another, the transition of dispersed cells to planktonic cells may be more subtle when the cells
7 remain in the same location. Hence, it is important to take note of the duration of the dispersal
8 process for each bacterial species and the time at which dispersed cells are harvested, as this may
9 affect the results of the biomarker assays or lead to false negatives in assays using dispersal
10 inducing treatments [AU: Edits to this paragraph OK?].

11 *In vivo* biofilm dispersal. As a control, the *P. aeruginosa* PAO1/pJN105 biofilms on the implants
12 are treated with only 0.9% NaCl with L-arabinose. This will be no activation of the *yhjH* PDE
13 and avoid complete biofilm dispersal. However, there will still be basal but very low levels of
14 biofilm dispersal being detected in the CFU ml⁻¹, which is physiologically relevant for any
15 bacterial biofilm. Raw data from animal studies will be statistically analyzed. A minimum of 8-
16 10 mice is required by statistics (Chi-Square (X²) or Regression) for each group in each
17 experiment. Animals are randomly divided into two groups (control & test).

19 **Materials**

20 **Reagents:**

21 ***In vitro* dispersal assays:**

22 LB Broth Miller (BD Difco, Cat. No. 244620)

- 1 LB Agar (BD Difco, Cat. No. 240110)
- 2 Ammonium sulfate (Merck, Cat. No. 1.01217)
- 3 Sodium phosphate dibasic (Calbiochem, Cat. No. 567547)
- 4 Potassium dihydrogen phosphate (Merck, Cat. No. 1.04873)
- 5 Sodium chloride (Merck, Cat. No. 06404)
- 6 Magnesium chloride hexahydrate (Merck, Cat. No. 1.05833)
- 7 Calcium chloride dihydrate (Merck, Cat. No. 1.02382)
- 8 Iron (III) chloride hexahydrate (Merck, Cat. No. 1.03943)
- 9 D-(+)-Glucose (Sigma Aldrich, Cat. No. G5767)
- 10 Casamino acid (BD Bacto, Cat. No. 223050)
- 11 L-(+)-Arabinose (Sigma Aldrich, Cat. No. A3256)
- 12 Crystal violet solution (Sigma Aldrich, Cat. No. HT90132)
- 13 Ethanol Absolute (Merck, Cat. No. 00983)
- 14 Gentamycin sulfate USP grade (MP, Cat. No. 190057)
- 15 Colistin sulfate salt (Sigma, Cat. No. C4461)
- 16 Ammonium acetate (Sigma, Cat. No. A1542)
- 17 Perchloric acid (70% v/v) (Sigma, Cat. No. 311421)
- 18 Potassium bicarbonate (Sigma, Cat. No. 431583)
- 19 Methanol (Sigma, Cat. No. 34860)

- 1 Sodium hydroxide (Sigma, Cat. No. 221465)
- 2 C-di-GMP (Invivogen, Cat. No. 61093-23-0)
- 3 ***In vivo* dispersal assays:**
- 4 Blue agar plates (State Serum Institute, Denmark, Cat. No. 694)
- 5 Milli-Q ddH₂O, sterile
- 6 Hypnorm® (0.315 mg ml⁻¹ fentanyl citrate and 10 mg ml⁻¹ fluanisone) (Roche)
- 7 Midazolam (5 mg ml⁻¹) (Roche)
- 8 Pentobarbital (200 mg ml⁻¹) with lidocainhydrochlorid (20 mg ml⁻¹) (DAK Gesundheit, Germany)
- 9 Bupivacaine (5 mg ml⁻¹) (SAD, Denmark, Cat. No. CAS 18010-40-7)
- 10 Buprenorphinum 0.3 mg ml⁻¹ (Temgesic®, Schering-Plough Europe, Brussels, Belgium)
- 11 CRITICAL: light-sensitive, store in the box
- 12 Sodium hypochlorite (NaClO) solution (13%) (vol/vol)- Bleach (Applichem, cat. no. A1647)
- 13 CAUTION: Alkaline, may cause skin irritation; wear protective clothes, gloves and goggles
- 14 LB Broth Miller (BD Difco, Cat. No. 244620)
- 15
- 16 **Bacterial strains and plasmids**
- 17 Bacterial strains and plasmids used in this study are listed in Table 1.
- 18 **Table 1. Bacterial strains and plasmids.**

	Relevant characteristics	Reference
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Strain		
PAO1	Prototypic non-mucoid wild-type strain	47
PAO1/ <i>p_{BAD}-yhjH</i>	Gentamycin resistance (Gm^r); PAO1 containing the <i>p_{BAD}-yhjH</i> vector	21
PAO1/ <i>p_{BAD}-yhjH</i> / <i>p_{cdrA}-gfp</i>	Gm^r ; PAO1 containing the <i>p_{BAD}-yhjH</i> vector Carbenicillin resistance ($Carb^r$); PAO1 tagged by Tn7- <i>p_{cdrA}-gfp</i> vector	21
PAO1/ <i>p_{BAD}-yhjH</i> / <i>p_{pvdA}-gfp</i>	Gm^r ; PAO1 containing the <i>p_{BAD}-yhjH</i> vector Tetracycline resistance (Tc^r); PAO1 containing the <i>p_{pvdA}-gfp</i> fusion reporter	21
PAO1 ^a	Prototypic non-mucoid wild-type strain *	48
PAO1 ^a /pJN105	Gm^r ; PAO1 ^a containing the pJN105 vector	27
PAO1 ^a / <i>p_{BAD}-yhjH</i>	Gm^r ; PAO1 ^a containing the <i>p_{BAD}-yhjH</i> vector	27
Plasmid		
pJN105	Gm^r ; broad-host-range vector carrying the <i>araBAD</i> promoter	49

p_{BAD} - <i>yhjH</i>	Gm ^r ; pJN105 carrying the <i>yhjH</i> gene	21
p_{cdrA} - <i>gfp</i>	Ampicillin resistance (Ap ^r), Gm ^r ; pUCP22 carrying the p_{cdrA} - <i>gfp</i> fusion	41
p_{pvdA} - <i>gfp</i>	Tc ^r ; miniCTX vector carrying the p_{pvdA} - <i>gfp</i> fusion	42
* PAO1 and PAO1 ^a are nonmucoid wild-type strains from two different sources.		

1

2 **Animals**

3 Female BALB/c mice (8-9 weeks of age, Taconic M&B A/S, Ry, Denmark).

4 **CRITICAL:** Mice are maintained on water and standard mouse chow at liberty for 14 d before
5 the experiment.

6 **CAUTION:** All animal studies should be performed in accordance with Institutional and
7 National guidelines and regulations. The animal experiments are performed in accordance to the
8 European Convention and the Directive for the Protection of Vertebrate Animals used for
9 Experimental and Other Scientific Purposes and the Danish law on animal experimentation, with
10 the authorization and approval from the National Animal Ethics Committee of Denmark (The
11 Animal Experiments Inspectorate, dyreforsogstilsynet.dk) under the permit number 2010/561-
12 1817. The reporting of the *in vivo* protocol has also adhered to the ARRIVE guidelines⁵⁰.

13 **Equipment:**

14 ***In vitro* dispersal assays:**

- 1 1 µl inoculation loop (Nunc) (Cat. No. I7773)
- 2 14 ml Round Bottom Snapcap Tubes (BD Biosciences)
- 3 Nunc Multi Dish 24 wells (Nunc Delta Si)
- 4 Nunc Tc Microwell 96F (SI W/LID Nunc Delta Si)
- 5 Tecan Infinite M200 Pro microplate reader
- 6 Microscopic slides, Twin End Frosted, 1.0 – 1.2 mm, Ground Edges (Biomedial Holdings)
- 7 Microscope Cover Glasses (Marienfeld Laboratory Glassware)
- 8 Zeiss Observer, ZI Inverted microscope (63X oil immersion lenses, Brightfield, Alexa Fluor 489
9 excitation/ emission filter)
- 10 Probe tip ultrasonicator (Sonics SM VibraCell CVX750)
- 11 Vacuum concentrator (SpeedVac)
- 12 High-performance liquid chromatography (Agilent, USA)
- 13 Reverse-phase C₁₈ Targa column (2.1 x 40 mm, 5 µm) (Cat. No. TR-0421-C185)
- 14 Bradford Protein Assay (Bio-Rad) (Cat. No. 500-0001)
- 15 ***In vivo* dispersal assays**
- 16 15 ml centrifuge tubes (TPP, cat. no. 91015)
- 17 50 ml centrifuge tubes (TPP, cat. no. 91050)
- 18 BD Microlance™ 3 needles (BD)
- 19 BD Plastipak™ syringes (BD)

- 1 LP reptiles vivarium heating mat, 1200 x 280 mm (LP Racks)
- 2 GraphPad software, Inc. (San Diego, USA, version 5.0)
- 3 Nasal scissors (B. Braun Aesculap, Cat. no. OK365R),
- 4 Durogrip needleholder (B. Braun Aesculap, Cat. No. BM065R)
- 5 Tweezers (B. Braun Aesculap)
- 6 Suture, Ethicon Vicryl, 3-0, FS-2 (Johnson-Johnson Intl.)
- 7 Minisart filter with pore size 0.2 μm (Sartorius, Vivascience AG)
- 8 IKA® Mixing Orbital shakers, KS 501 digital (IKA)
- 9 Centrifuge (e.g. Sorvall RC6)
- 10 Spectrophotometer (e.g. Shimadzu UV-1601)
- 11 50 ml and 250 ml Erlenmeyer flasks (sterile)
- 12 Silicone tube, length 2mm, cut in half lengthwise (inner diameter, 4 mm; outer diameter, 6 mm;
- 13 Ole Dich, Denmark) (Implants)
- 14 Ultrasound bath (Bransonic model 2510; Branson Ultrasonic Corp)
- 15 1 ml syringe (300013, BD Biosciences, San Jose, CA, US)
- 16 2 ml syringe (300186, BD Biosciences, San Jose, CA, US)
- 17 5 ml syringe (300186, BD Biosciences, San Jose, CA, US)
- 18 Cell strainer, 40 μm -pore-size (BD Biosciences, San Jose, CA, US)
- 19 **Reagent setup**

- 1 ABTGC: consists of A10, BT, glucose, casamino acids and other chemicals (all stored at room
- 2 temperature unless stated otherwise), as follows:
- 3 100 ml of A10
- 4 15.1 mM Ammonium sulfate
- 5 33.7 mM Sodium phosphate dibasic
- 6 22.0 mM Potassium dihydrogen phosphate
- 7 0.05 mM Sodium chloride
- 8 900 ml BT
- 9 1 mM Magnesium chloride hexahydrate
- 10 100 μ M Calcium chloride dihydrate
- 11 1 μ M Iron (III) chloride hexahydrate
- 12 10 ml 20% D-(+)-Glucose (stored at 4 °C)
- 13 10 ml 20% Casamino acid
- 14 ABTGC can be stored in room temperature for up to 6 months.
- 15
- 16
- 17 25% w/v arabinose: Dissolve 25 g of arabinose in 100 ml ddH₂O (filter sterilize with 0.2 μ m
- 18 filter (Nalgene, USA) and store at 4 °C for up to 6 months).
- 19

1 0.9% w/v NaCl: Dissolve 0.9 g of NaCl in 100 ml ddH₂O (filter sterilize with 0.2 µm filter
2 (Nalgene, USA) and store at room temperature for up to 6 months)

3

4 0.01% v/v crystal violet: Mix 10 µl of crystal violet in 100 ml ddH₂O (store at room temperature
5 for long periods of time).

6 CAUTION: Crystal violet is toxic and causes eye irritation. Treat the compound in chemical
7 hood, wearing the appropriate personal protection equipment (PPE).

8

9 1 mM ice cold ammonium acetate: Mix 77.1 mg of ammonium acetate in 100 ddH₂O. Store at 4
10 °C for up to 6 months.

11

12 0.6 M perchloric acid: 70% perchloric acid is approximately 11.6 M. Add 5.17 ml of perchloric
13 acid to ddH₂O to reach a total volume of 100ml. Store at room temperature for up to 1 month.

14 CAUTION: perchloric acid is highly corrosive and volatile. Treat the compound in chemical
15 hood, wearing the appropriate personal protection equipment (PPE). Always add acid to ddH₂O.

16

17 2.5 M potassium bicarbonate: Dissolve 2.5 g in 10 ml ddH₂O. Store at room temperature for up
18 to 6 months.

19

20 1 M sodium hydroxide: Mix 4 g in 100 ml ddH₂O. Store at room temperature for up to 1 month.

CAUTION: sodium hydroxide is highly corrosive. Treat the compound in chemical hood, wearing the appropriate personal protection equipment (PPE).

10 mM ammonium acetate in ddH₂O: Mix 771 mg of ammonium acetate in 100 ml ddH₂O. Store at room temperature for up to 6 months.

10 mM ammonium acetate in methanol: Mix 771 mg of ammonium acetate in 100 ml methanol. Store at room temperature for up to 1 month.

CAUTION: methanol is highly toxic. Treat the compound in chemical hood, wearing the appropriate personal protection equipment (PPE).

1 mM c-di-GMP in 1mM ammonium acetate: Dissolve 1 mg of c-di-GMP in 1.381 ml 1mM ammonium acetate to prepare 1 mM c-di-GMP. Store the stock at -20 °C for up to 1 month. Further dilute 1000X in 1mM ammonium acetate to get 1 nM c-di-GMP freshly before use.

Analgesia and anesthesia:

Mix one part Hypnorm with three parts of sterile water. Then add one part midazolam (5 mg ml⁻¹). Mix gently.

CRITICAL: Prepare mixture freshly before use.

2% w/v L-arabinose: Dissolve 2 g of L-arabinose in 100 ml 0.9% NaCl and sterile filter the solution. The solution should be heated to body temperature before injection into the mice.

Solvent A for HPLC: 10 mM ammonium acetate in water (made freshly before use). Solvent B for HPLC: 10 mM ammonium acetate in methanol (made freshly before use).

EQUIPMENT SETUP

HPLC Setup: [AU: Please explain the setup here, mentioning first the analytical column already in the EQUIPMENT section, the injection loop, the flow rate and the detecting wavelength, then place text about the guard columns after this].

HPLC Elution gradient: 0 to 8 min with 1% B; 8 to 14 min with 15% B; 14 to 16 min with 25% B; 16 to 24 min with 90% B; 24 to 32 min with 100% B; 32 to 40 min, 1% B; 40 to 42 min, 1% B, as shown below:

Time (min)	% B
0-8	1
8-14	1-15
14-16	15-25
16-24	25-90
24-32	90-100

32-40	100-1
40-42	1

[AU: Edit OK? Inclusion of table for added clarity]

HPLC Column rinse elution gradient: 0 to 58 min, 100% B; 58 to 70 min, 1% B [AU: Do you mean "0 to 58 min, 1 to 100 % B; 58 to 70 min, 100 to 1 % B"? This is slightly ambiguous as written, is it worth editing this for clarity?]

Procedure

***In vitro* assay: Preparation of inoculum (Timing: 10 min + 12 h incubation)**

- 1 Inoculate PAO1/p_{BAD}-y_{hjH} (Gentamicin resistant) from – 80 °C freezing cultures by using a 1 µl inoculation loop into 2 ml LB with 30 µg ml⁻¹ gentamicin in a 12 ml culture tube and incubate in temperature-regulated shaker set at 200 rpm, 37 °C for 12 h.

***In vitro* assay: Growing biofilm or planktonic cells (for comparison to dispersed cells as a control)**

- 2 Dilute the overnight culture 100 X with ABTGC. Mix well.
- 3 Transfer 1 ml of diluted culture into each well of a 24-well plate. Prepare 12 wells for PAO1/p_{BAD}-y_{hjH}.
- 4 Incubate the plate using option A to grow biofilm or option B to grow planktonic cells.

A. Growing biofilm (Timing: 30 min + 24 h incubation)

- i. Incubate plate at 37°C for 24 h to allow biofilm growth.

? Troubleshooting

B. Growing planktonic cells(Timing: 7 h)

- i. Incubate plate at 37°C, 200 rpm shaking for 7 h. This will allow the planktonic cells to grow to late logarithmic phase.

***In vitro* assay: Dispersal of biofilm (Timing: 30 min + 5 h incubation)**

- 5 Remove and discard the liquid culture from the 24-well plate from Step 4A and wash the wells three times with 1 ml 0.9% NaCl per well using a 1 ml Eppendorf pipette.

CRITICAL STEP: a ring of biofilm forms and attaches onto the wall of the well at the air-liquid interface. Floating aggregates and planktonic cells are removed during washing, leaving behind the ring of biofilm which is firmly attached to the wall of each well.

- 6 Add 1 ml of fresh ABTGC with 0.25% w/v L -arabinose to PAO1/ p_{BAD} - y_{hjH} biofilms to initiate dispersal. ABTGC with 0% w/v L -arabinose is used as control, which will not disperse biofilms.

- 7 Incubate for 5 h at 37 °C.

CRITICAL STEP: To enable downstream experiments on dispersed cells, we recommend allowing between 3 to 5 h to disperse biofilms. Longer incubation may result in dispersed cells converting to planktonic state.

? Troubleshooting

***In vitro* assay: Measurement of OD600 nm and colony forming units (CFU ml⁻¹) of dispersed cells (Timing 2 h + 18 h incubation)**

- 8 Transfer 1 ml culture containing dispersed cells from Step 7 to a well in a new 24-well plate. Retain the existing plate for use in Step 14 [AU: How should the plate be stored in the meantime?]
- 9 Measure the OD_{600 nm} of the dispersed cells with the Tecan Infinite M200 PRO microplate reader.
- 10 Dilute 20 µl of dispersed cells in 180 µl 0.9% NaCl, in triplicate. Mix well and dilute each serially to 10⁻⁸ dilution in 0.9% NaCl.
- 11 Spread 100 µl of the diluted cultures (10⁻⁶ to 10⁻⁸ dilutions) on LBA plates in triplicate and allow to dry.
- 12 Incubate the plates for 18 h at 37 °C.
- 13 Count the number of colonies formed on each LBA plate. Choose the plates with a minimum of 30 and maximum 300 colonies. and calculate the CFU ml⁻¹ as follows:
- CFU ml⁻¹ = (Average number of colonies [AU: Does this mean the average taken from the triplicate plates for a specific dilution]) X (degree of dilution that is plated) X (10 to allow for the volume plated). CRITICAL STEP: Plates with less than 30 colonies are too diluted and statistically insignificant for counting. Plates with more than 300 colonies are too concentrated and too crowded to count single colonies.

***In vitro* assay: Biofilm staining with crystal violet (Timing: 30 min)**

- 14 Wash the biofilm remaining in the well in Step 8 three times with 1 ml 0.9% NaCl.

Critical STEP: Any floating aggregates must be washed away thoroughly, leaving behind the true biofilm that attached to the wall of each well at the air-liquid interface. This prevents non-specific crystal violet staining of the aggregates. Moreover, care must be taken not to **scrape [AU: Is this what you mean?]** the biofilm attached to the wall of the well.

15 Stain the biofilm with 1ml 0.01% crystal violet for 10 min. Pour away the crystal violet

CAUTION: crystal violet is toxic.

16 Wash the biofilm three times with 1 ml 0.9% NaCl.

17 Add 1 ml of 70% ethanol to dissolve the stained biofilm for 10 min.

18 Measure the OD 590 nm with Tecan microplate reader to quantify the intensity of crystal violet stain. The linear range of the microplate reader is ~0.1 to ~1.0. If the value exceeds 1.0, dilute the samples by five times with 70% ethanol and measure OD 590 nm again.

***In vitro* assay: Quantification of c-di-GMP in dispersed and planktonic cells by HPLC (universal biomarker) (Timing 2 h)**

19 Harvest 11 ml of dispersed cells and 11ml of planktonic cells by combining the cultures from 11 wells of the 24-well plate prepared in Step 4A or 4B, respectively. Aliquot 1 ml of cells and save for quantification of protein concentration in **Steps 33-34 [AU: Edit correct?]**. Centrifuge the remaining cells at 13000 g for 3 min and **remove supernatant [AU: Do you discard the supernatant?]**.

20 Wash cell pellet with 10 ml 1 mM ammonium acetate and centrifuge cells at 13000 g for 3 min to remove supernatant and discard. Repeat twice.

21 Re-suspend pellet in 300 µl of 0.6 M perchloric acid.

CAUTION: Perchloric acid is corrosive and toxic.

22 Lyse bacterial cells with the probe tip ultrasonicator (amplitude 30%; 5 s ON, 5 s OFF) for 1 min in ice-cold water bath.

CAUTION: As probe sonicator delivers high-energy input to the samples, do not touch the vibrating tip during use.

CRITICAL STEP: Ensure that bacterial cells are totally lysed with reduction in optical density [AU: Please clarify what you mean here? How can readers tell when the samples are totally lysed? I assume that you mean that lysis will result in a reduction of optical density so you could say “Measure optical density to check that that cells are totally lysed; cell lysis results in a reduction in optical density (from xx to xx, for example).”] to allow all c-di-GMP molecules to be released from the cell debris. Ensure that the samples are always put in an ice-cold bath to prevent heat from the sonication degrading the c-di-GMP molecules.

23 Incubate samples on ice for 30 min.

24 Centrifuge samples at 15000 g for 5 min to remove cell debris and transfer supernatant to a new 1.5 ml Eppendorf tube.

25 Add 30 μ l of 2.5 M KHCO_3 to supernatant to neutralize the perchloric acid and incubate on ice for 10 min.

CRITICAL STEP: Bubbles may form during the neutralization; avoid spillage of samples by... [AU: Please explain how you avoid sample spillage?].

CRITICAL STEP: Ensure that the neutralization of acid is complete by incubating on ice for 10 min.

26 Centrifuge samples at 15000 g for 5 min to remove KClO_4 precipitate and transfer supernatant to new 1.5 ml Eppendorf tube.

27 Filter the supernatant samples with a 0.2 μ m syringe filter to remove any remaining particulates that can potentially block the HPLC column.

28 Prepare fresh 0, 1, 2, 5, 10 and 20 μM pure c-di-GMP standards in 1 mM ammonium acetate.

29 Load the standards and samples in the HPLC instrument with injection volumes of 10 μl and the detection wavelength of 254 nm. Perform a parallel-run for each cell sample (A) and cell sample plus 1 nM standard c-di-GMP solution (A+1 μM c-di-GMP).

CRITICAL STEP: Run a standard c-di-GMP solution (1 μM) after every six cell samples to check the column performance.

30 Run the samples through the Reverse-phase C_{18} Targa column (2.1 x 40 mm, 5 μm), using the solvents and elution gradient as detailed in the Reagent Setup and Equipment Setup, respectively at a flow rate of 0.2 ml min^{-1} .

CRITICAL STEP: Filter all solvents through the 0.2 μm membrane before injecting into the HPLC to prevent clogging of HPLC column.

CRITICAL STEP: A column rinse should be performed after every six samples using a rinse recipe as detailed in the Equipment Setup. More than 20 cell samples can be run through the same column without significant peak shift and broadening. The c-di-GMP extract may contain protein residue, polysaccharide and inorganic salt which can increase the internal pressure and block the HPLC column easily. A guard column can be placed

between the injector and the HPLC column. Guard columns are similar to, yet shorter in length and cheaper than analytical columns so they protect the HPLC column from strongly retained impurities. This reduces contamination and blocking of the analytical column, but may result in poorer separation. Guard columns can be replaced after 20 to 30 samples. [AU: Please add these to the Equipment section with Supplier details and if, necessary, explain how to add them to the HPLC setup?].

31 Compare the time of elution of 10 μ M pure c-di-GMP standard to that of the samples. In

our setup, the retention time of c-di-GMP is around 14.0 min [AU: Edit OK?]. To

further confirm that the peak at the time of elution can be attributed to c-di-GMP, there should be an increment of the peak area in cell sample plus 1 nM standard c-di-GMP solution (A+1 nM c-di-GMP), as compared to cell sample (A).

32 Calculate the relative concentration of c-di-GMP in the samples using the peak area at the time of elution. From the peak areas of c-di-GMP standards, prepare a standard curve by plotting peak areas (y-axis) over c-di-GMP concentration (x-axis). Use the peak area of each sample to infer the c-di-GMP concentration in each sample.

33 For total protein quantification, re-suspend the saved 1 ml of cells from Step 19 in 1 ml 1 M NaOH. Incubate samples at 95 °C for 5 min to lyse cells.

CAUTION: Avoid direct contact with NaOH and personal exposure to high temperature.

34 Measure protein concentration using the Bradford assay (Bio-Rad), according to the manufacturer's instructions.

35 Normalize the relative concentration of c-di-GMP in the samples calculated in Step 32 to the total protein concentration from Step 34. Compare the relative concentration of c-di-GMP in dispersed cells to planktonic cells.

?TROUBLESHOOTING

***In vitro* assay: Microscopy of p_{cdrA} -gfp- tagged strains to compare transcriptional expression levels of *cdrA* gene between planktonic and dispersed cells (*P. aeruginosa*-specific biomarker) (Timing 1 h)**

36 Prepare PAO1/ *p_{cdrA}-gfp*- tagged strains as described in Steps 1-7 and transfer 6 µl of dispersed cells (and planktonic cells as control) onto microscope slides and mount on the coverslip.

37 Observe and capture images of the cells with Zeiss Observer, ZI Inverted microscope, at 630 X magnification (oil immersion) with bright field and Alexa Fluor 469 nm filter set (1.5 sec exposure).

38 Measure the bacterial cell fluorescence using ImageJ.

? Troubleshooting

***In vitro* assay: Quantification of *p_{pvdA}-gfp* - tagged strains to compare transcriptional expression levels of *pvdA* gene between planktonic and dispersed cells (*P. aeruginosa*-specific biomarker) (Timing: 30 min)**

39 Prepare PAO1/ *p_{pvdA}-gfp*- tagged strains as described in Steps 1-7 and add 200 µl of dispersed cells (and planktonic cells as control) into each well of a 96-well plate. Prepare triplicate wells/ strain.

40 Measure the OD600 nm and GFP fluorescence (Excitation: 485nm/ Emission: 535 nm) using the Tecan microplate reader.

41 Calculate the relative fluorescence intensity by dividing GFP values with OD 600 nm values.

? Troubleshooting

***In vitro* assay: Quantification of pyoverdine in dispersed cells (*P. aeruginosa*- specific phenotype) (Timing: 30 min)**

42 Prepare PAO1/ *p_{pvdA}-gfp*- tagged strains as described in Steps 1-7 and add 200 µl of dispersed cells into each well of the 96-well plate. Prepare triplicate wells/ strain.

43 Measure the OD₆₀₀ and pyoverdine fluorescence (Ex 400 nm/ Em 460 nm) using the Tecan microplate reader.

44 Normalize the pyoverdine levels by dividing pyoverdine fluorescence with OD₆₀₀.

In vivo mouse implant model: Preparation of bacterial biofilm on implant (Timing 4 d)

45 Prepare implants from silicone tubes (inner diameter 4 mm; outer diameter 6 mm; length 1mm). Make a minimum of one implant per mouse and sterilize the implants by autoclaving for 20 mins at 121 °C.

CRITICAL STEP: prepare four extra implants as reserves and for CFU ml⁻¹ counting of implant as control at 0th hour.

PAUSE POINT: The implants are prepared at any time before the start of the experiment and can be stored at room temperature for long periods of time.

46 Streak PAO1/*p_{BAD}-yhjH* and PAO1/pJN105 bacteria from a freezer stock by using a 1 µl inoculation loop onto a blue agar plate and incubate overnight at 37 °C for single colonies to grow. The agar plate could be stored at 4 °C for up to 3 weeks.

CRITICAL STEP: The blue agar plate is a modified Conrad Drigalski medium selective for Gram-negative rod bacteria, such as *P. aeruginosa*⁵¹. Other selective plates should be used for other bacterial species. However, LB plates will also suffice in the growing of colonies and counting of CFU ml⁻¹ of bacteria.

47 Pick 1 bacterial colony from the agar plate and inoculate as described in Step 1.

48 Harvest and wash cells by centrifuging 2 ml of bacterial culture in 2 ml tubes at 3000 *g* for 10 min at 20 °C. Remove the supernatant with a pipette and discard, and [AU: Edit

correct?] re-suspend the bacterial pellet in 2 ml 0.9% NaCl.

49 Measure the OD₆₀₀ of the cell suspension and dilute in 10 ml 0.9 % NaCl to an OD₆₀₀ = 0.1.

50 Place up to 10 implants together into a 50 ml Erlenmeyer flask with 10 ml of the re-suspended culture with OD₆₀₀ of 0.1.

CRITICAL STEP: Ensure consistency by placing same number of implants and volume of 0.9% NaCl in each Erlenmeyer flask for each experiment. Push the implants with a pipette to the bottom of the flask to ensure that all the implants do not float and are submerged for even growth of biofilm on the implants.

51 Incubate the implants at 37 °C for 20 h, shaking at 110 rpm to allow biofilm growth on the implants.

52 Remove each implant with biofilm from the flask with forceps and dip the implant gently once in a well containing 2 ml 0.9% NaCl in a 24-well plate to wash away the planktonic bacteria. Place each implant into a new well of a 24-well plate containing 2 ml 0.9 % NaCl in each well [AU: Edits correct?].

53 Use 1 implant as a 0th hour control to determine the number of biofilm bacteria formed on the implant. Place this implant into a 2 ml tube containing 1 ml 0.9% NaCl and sonicate it in an ultrasound bath with ice bath water for 10 min (a 5-min degas step followed by a 5-min sonic step). The ultrasonic sonication will effectively and efficiently dislodge the biofilm cells from the implant. The degas step will expel trapped air bubbles in the bath

water as trapped air can impede sonication and decrease the effectiveness of biofilm disruption.

CRITICAL STEP: Ensure consistency by using the same volume of bath water, duration of degas and sonication for each experiment. Monitor the temperature of the bath water continuously with a thermometer and check it remains at around 4 °C and replenish ice if required. Increase in temperature by sonication can affect the viability of the biofilm cells.

54 Prepare serial dilutions (10-fold) of the bacterial suspension [AU: in 0.9% NaCl?] and plate the 10^{-3} , 10^{-4} , 10^{-5} dilutions on blue agar plates [AU: What volume of dilution is plated?].

55 Incubate plates overnight at 37 °C.

56 Count the bacterial colonies on the plates. Choose the plates with a minimum of 30 and maximum 300 colonies. CRITICAL STEP: Plates with less than 30 colonies are too diluted and statistically insignificant for counting. Plates with more than 300 colonies are too concentrated and too crowded to count single colonies.

57 Calculate the CFU ml⁻¹ per implant as described in Step 13 [AU: Please check the equation in Step 13 is correct – depending on the volume plated in Step 54?]. This represents the number of biofilm bacteria formed on the implant without placement in the mouse.

? Troubleshooting

***In vivo* mouse implant model: Surgical insertion of implant intraperitoneally in mice**

(Timing: 30 - 32 h)

58 Anesthetize the mice by subcutaneous injection at the groin with Hypnorm/midazolam (see Reagent Setup).

CRITICAL STEP: Ensure that university/ institution animal ethics and care regulations are followed. Check that the mice have lost consciousness by pinching the limbs gently and observing no twitching response from the mice.

59 Fix the anesthetized mouse on its back and sterilize the incision area with 70% (v/v) ethanol. Make an incision of 1 cm in the groin area by cutting the skin and the underlying peritoneal wall to gain access to the peritoneal cavity with surgical scissors.

CAUTION: Surgical tools are sharp equipment. Care must be taken in handling them.

CRITICAL STEP: Check that the peritoneal cavity has been accessed with both the skin and peritoneal wall having been incised. This prevents the implant from being inserted incorrectly between the skin and the peritoneal wall.

60 Insert the implant into the peritoneal cavity by holding the incision open with a forcep and placing the implant near the intestines gently with another forcep. Suture the incision with silk tightly.

CRITICAL STEP: Ensure that the implant does not tangle with the intestines. Suture both the peritoneal wall and the subcutaneous (s.c) skin.

61 Sterilize all surgical equipment with 70% (v/v) ethanol after each mouse.

62 Inject 750 μ l of 0.9% NaCl in the neck area of each mouse with a 25 G needle, to prevent dehydration of the mouse.

CRITICAL STEP: 0.9% NaCl should be pre-warmed to 37 °C before injection.

63 Drip a drop of bupivacaine with a 18 G needle onto the incision to manage post-operative pain of each mouse.

64 Place the mice lying on their side (incision side facing upward) on the bedding in clean cages and cover them with a paper napkin for post-operative care. Place the cage on a heating pad, with temperatures no higher than 28 °C.

65 Check the health of the mice and check that they recover from anesthesia after 6 h post surgery [AU: Edit OK?]. Inject 100 µg kg⁻¹ of buprenorphinum subcutaneously with a 25 G needle to manage post-operative pain.

66 Inject 100 µg kg⁻¹ of buprenorphinum subcutaneously with a 25 G needle to manage post-operative pain at 12 h post surgery. Check the health of the mice at least once every day.

After insertion of implant with *P. aeruginosa* biofilms, the mice will develop symptoms typical of infections, such as reduced activity, fever, hypothermia and/or dehydration. Administer fluid therapy with 300 – 500 µl glucose and 0.9% NaCl to mice with dehydration, inability to eat or drink, or immotility. Provide warm-pad of not more than 28 °C to animals with hypothermia. Euthanize any mice with severe dyspnea, severe bleeding, spasm or severe hypothermia by intraperitoneal injections with Pentobarbital (200 mg ml⁻¹ at 10 ml kg⁻¹ of body weight) immediately. Use the live mice for the downstream steps, and dispose of the dead mice appropriately in the biohazard bag. CAUTION: Ensure that local animal care regulations are followed.

67 Maintain the mice for 24 h to allow biofilms in the implant to develop an infection.

?TROUBLESHOOTING

68 Inject 200 μ l of 2% (w/v) L -arabinose solution at the implant site with a 25 G needle at 24 h and 26.5 h post-insertion of implant. This will induce PAO1/*p_{BAD}-yhjH* biofilm dispersal when the L -arabinose activates the inducible expression of YhjH which degrades c-di-GMP. As control, PAO1/pJN105 is not affected by the addition of L -arabinose.

CRITICAL STEP: The arabinose solution should be heated to 37 °C before injection.

69 Euthanize the mice by injecting Pentobarbital (200 mg ml⁻¹ at 10 ml kg⁻¹ of body weight) and lidocaine hydrochloride (20 mg ml⁻¹ at 10 ml kg⁻¹ of body weight) at the groin area with a 25 G needle at 29 h post-insertion of implant.

CRITICAL STEP: Ensure that local animal care regulations are followed. Check that the mice have died by pinching the limbs gently and observing no twitching response from the mice.

?TROUBLESHOOTING

70 Fix the dead mouse on its back firmly and carefully. Spray the incision site with 70% (v/v) ethanol to reduce contamination during the removal of the implant.

71 Cut open the incision site with the surgical scissors. Remove the implant carefully from the surrounding organs and cut any attached tissue away from the implant. Place the implant into a 2ml tube containing 1 ml 0.9% NaCl with forceps. Place the implants on ice.

CRITICAL STEP: The implants should be kept on ice for at most 2 to 3 h. Hence, it is necessary to process the implants as soon as possible after the mice have been sacrificed.

72 Suture the incision with silk firmly so that the incision site is closed tightly.

CRITICAL STEP: Suture both the peritoneal wall and the subcutaneous skin. The incision site must be closed tightly to prevent spillage of 0.9% NaCl described in Step 73.

73 Inject 2 ml 0.9% NaCl with a 25 G needle near the incision site and press gently on the peritoneal cavity area from the mouse exterior. This is to flush the bacterial cells that have dispersed from the implant and remained in the peritoneal cavity.

74 Re-open the peritoneal cavity gently by cutting the incision site with the surgical scissors. Retrieve the dispersed bacteria in 0.9% NaCl using a disposable pipette and transfer it to a 2 ml tube. Place the samples on ice.

CRITICAL STEP: The 0.9% NaCl should be kept on ice for at most 2 to 3 h. Hence, it is necessary to process the cells as soon as possible after the mice have been sacrificed.

75 Using surgical scissors cut out the spleen and remove with forceps [AU: Edit OK?].

Place the spleen in 2 ml 0.9% NaCl on ice. The spleen contains dispersed bacterial cells from the bloodstream during its filtering of the blood.

CRITICAL STEP: The spleen should be kept on ice for at most 2 to 3 h. Hence, it is necessary to process the spleens as soon as possible after the mice have been sacrificed.

76 Sterilize the surgical equipment between each mouse with 70% (v/v) ethanol.

CRITICAL STEP: Samples and tissues must be transferred in a sealed container during transport from the animal facility to the laboratory, for downstream evaluation.

***In vivo* mouse implant model: Quantification of CFU ml⁻¹ of bacteria from implant, peritoneal cavity and spleen (Timing: 24 h)**

77 Implant from Step 71: Place the centrifuge tubes in an ultrasound bath with ice water as described in Step 53.

78 Spleen from Step 75: Force the spleen through a 40 µm-pore size cell strainer using the plunger of a 1-ml syringe, to homogenize the spleen and release single bacterial cells.

79 Serially dilute, plate dilutions and calculate CFU ml⁻¹ for samples of implant from Step 77, spleen from Step 78 and 0.9% NaCl from peritoneal cavity from Step 74, as described in Steps 54 to 57.

80 Employ Chi-Square (X²) or Regression analysis to calculate the statistical significance of differences in CFU ml⁻¹ between samples treated with and without L-arabinose, using statistical analysis software such as SPSS Statistics 2015. P-values of less than 0.05 will signify statistical significant difference between samples.

Troubleshooting

Please refer to Table 2.

Table 2. Troubleshooting.

Step No.	Problem	Possible Reason	Solution
<i>In vitro</i> assay			
4Ai	Biofilm not formed	Low inoculum concentration or short period of incubation	Increase inoculum concentration and duration of incubation

7	Biofilm not dispersed	Concentration of arabinose is too low or dispersal duration is too short	Increase arabinose concentration and dispersal duration
35	Low levels of c-di-GMP	Low cell numbers in samples	Harvest higher quantity of cells
		Incomplete lysis of cells	Increase volume of perchloric acid or increase duration of sonication
38	<i>p_{cdrA}-gfp</i> levels have increased	Washing step to remove planktonic cells before dispersal treatment not complete	Wash the biofilm free from planktonic cells thoroughly before dispersal treatment
41	<i>p_{pvdA}-gfp</i> levels have increased	Dispersed cells reverted to planktonic state	Decrease duration of dispersal

<i>In vivo</i> assays			
57	Low bacterial CFU ml ⁻¹ grown on implant before insertion into mouse	Poor growth of biofilm on implant or low inoculum density	Regrow the biofilm on implants with high inoculum density. It is desired to obtain a minimum of $\sim 2.5 \times 10^6$ CFU ml ⁻¹ per implant.
67	Mouse cleared the biofilm infection from the implant too rapidly (usually 1 to 2 days)	Poor growth of biofilm on implant or low inoculum density	Regrow the biofilm on implants with higher inoculum density. It is desired to obtain a minimum of $\sim 2.5 \times 10^6$ CFU ml ⁻¹ per implant.
69	Low survival rate of mice before sacrifice	Mice inoculated with implants containing high	Regrow the biofilm on implants with

		biofilm numbers	<p>suitable inoculum density. Usually, the mice will look sick within the initial 24 hour post-infection due to the administration of anesthetics and the surgical procedure.</p> <p>However, they should look quite healthy after 1d, but with a reduced degree of cleanliness.</p>
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1

2 **Timing**

3 *In vitro* dispersal assay:

4 Step 1 Preparation of inoculum (day 0): Timing 10 min + 12 h incubation

5 Steps 2 – 4A Growing biofilm (day 1): Timing 30 min + 24 h incubation

- 1 Step 4B Growth of planktonic cells as control to dispersed cells (day 2): Timing 7 h
- 2 Steps 5 – 7 Dispersal of biofilm (day 2): Timing 30 min + 5 h incubation
- 3 Steps 8 – 13 Measurement of OD600nm and colony forming units (CFU ml⁻¹) dispersed cells
- 4 (days 2 and 3): Timing 2 h + 18 h incubation
- 5 Steps 14 – 18 Biofilm staining with crystal violet (day 2): Timing 30 min
- 6 Steps 19 – 35 Quantification of c-di-GMP by HPLC (day 2): Timing 2 h
- 7 Steps 36 – 41 Fluorescence quantification of *p_{cdrA}-gfp* and *p_{pvdA}-gfp* - tagged strains (day 2):
- 8 Timing 1.5 h
- 9 Steps 42 – 44 Quantification of pyoverdine of dispersed cells (day 2): Timing: 30 min
- 10 *In vivo* dispersal assay:
- 11 Steps 45 – 57 Preparation of bacterial biofilm on implant (day 0): Timing 4 d
- 12 Steps 58 – 76 Surgical insertion of implant intraperitoneally in mice (day 4): 30-32 h
- 13 Steps 77 – 80 Quantification of CFU of implant, peritoneal cavity and spleen (day 6): 24 h

14

15 **Anticipated Results**

16 *In vitro* dispersal of biofilm and generation of dispersed cells:

17 After 5 h incubation with the dispersal agent (L-arabinose, Step 7), the biofilm will disperse into
18 the ABTGC medium, causing the rise in optical density (Fig. 3a) and CFU ml⁻¹ of dispersed cells
19 (Fig. 3b). The increased dispersal of biofilm corresponding to increasing arabinose concentration
20 implied that dispersal by genetic means is dose dependent. The biomass of the dispersed cells

measured with OD600 nm (Step 9) was comparable to the viable cell counts of dispersed cells, measured by means of CFU (Step 13; Fig. 3a and Fig. 3b). Correspondingly, the biomass of the biofilm, as measured by crystal violet, decreased during dispersal (Step 18; Fig. 3c).

C-di-GMP biomarker universal to dispersed bacterial cells:

As c-di-GMP is a ubiquitous secondary messenger in both Gram-positive^{52,53} and Gram-negative⁵⁴⁻⁵⁶ bacteria, direct detection of intracellular c-di-GMP levels is an important biomarker to differentiate dispersed cells from planktonic cells. When quantified by HPLC (Step 35), dispersed cells possessed lower c-di-GMP levels than planktonic cells (Fig. 4).

Biomarkers specific to *P. aeruginosa* dispersed cells:

Relative fluorescence quantification of cells with key markers *p_{cdrA}-gfp* and *p_{pvdA}-gfp* (Steps 38 and 41 [AU: edit correct?]) is shown in Fig. 5. These genetic reporters demonstrate that dispersed cells generated by this protocol express lower levels of *p_{cdrA}-gfp* (Fig. 5a) and *p_{pvdA}-gfp* (Fig. 5b), compared to planktonic cells.

Phenotypes specific to *P. aeruginosa* dispersed cells:

Our phenotypic assay showed lower pyoverdine levels in dispersed cells, compared to planktonic cells (Step 44, Fig. 6). We strongly recommend using planktonic cells as a negative control in the characterization of dispersed cells.

In vivo dispersal of Biofilm:

The group of mice that had implants pre-colonized with PAO1/*p_{BAD}-yhjH* and dispersed with L-arabinose (Step 68), had lower CFU ml⁻¹ on the implants (Step 79) compared to the group of mice with implants colonized with PAO1/pJN105 vector control (Fig. 7). Regarding the bacterial cells dispersed from the implant, the mice that had implants pre-colonized with PAO1/ *p_{BAD}*-

yhjH and dispersed with arabinose had a higher CFU ml⁻¹ of bacteria, both in the fluid from the peritoneal cavity flush and in the spleen, compared to mice with implants colonized with PAO1/pJN105 (Fig. 7). We have observed that after the induction of biofilm dispersal the number of PAO1/ *p_{BAD}-yhjH* bacteria in the spleens of the mice increased during the first 5 h. The mice did not suffer any adverse effects during the experiments and showed no signs of discomfort. Although the increasing number of PAO1/ *p_{BAD}-yhjH* bacteria in the spleen could be a concern since the presence of released bacteria could lead to sepsis and a fatal outcome for the mice, it is important to note that after 5 h, the bacterial count of PAO1/ *p_{BAD}-yhjH* in the spleen decreased to similar levels of the PAO1/pJN105 control.

Figures:

Figure 1. Schematic workflow of biofilm dispersal and evaluation of dispersed cells *in vitro*. (a) Basic overview of dispersal procedure. Planktonic cells are first grown to biofilm mode and then dispersed with a dispersal agent (Steps 1 to 7). Dispersed cells are then harvested for downstream purposes (Step 8). (b) Evaluation of dispersed cells. The dispersed cells are quantified by assessing optical density (OD₆₀₀) and CFU ml⁻¹ (Steps 9 to 13). The measurement of c-di-GMP levels by HPLC is used as a universal biomarker to compare planktonic and dispersed cells (Steps 19 to 35). The *p_{cdrA}-gfp* and *p_{pvdA}-gfp* biosensors are used as *P. aeruginosa*- specific transcript biomarkers of dispersed cells (Steps 36 to 41). *P. aeruginosa*- specific phenotypes of dispersed cells are also used to compare with planktonic cells (Steps 42 to 44).

Figure 2. Schematic workflow of biofilm dispersal and evaluation of dispersed cells *in vivo*. A biofilm is grown on an implant (Steps 45 to 51) and the implant is washed with 0.9% NaCl (Step

52). The BALB/C mouse is incised at the groin area (Steps 58 to 59) for insertion of implant into the peritoneum (Step 60). The incision site is sutured and the mouse is incubated for 24 h (Step 67). The implant biofilm is dispersed by injecting a dispersal agent into the incision site at the 24th and 26.5th h (Step 68). The mouse is sacrificed at 29th h (Steps 69 to 75), with the bacteria from the implant, peritoneal cavity around implant and spleen being extracted for CFU ml⁻¹ analysis (Steps 77 to 80).

Figure 3. Quantification of dispersed cells and biofilm after 5 h dispersal treatment in vitro [AU: Edit correct?]. (a) OD 600 nm of PAO1/p_{BAD}-yjhH dispersed cells with and without induction by L-arabinose (Step 9). (b) Viable cell count, in CFU ml⁻¹ of dispersed cells with and without induction by arabinose (Step 13). With the addition of L-arabinose, the OD 600 nm and CFU ml⁻¹ of dispersed cells increased. (c) Crystal violet stain of biofilm biomass after dispersal (Step 18) [AU: Step numbers added OK?]. The biomass of biofilm after dispersal decreased with L-arabinose induction, as compared to without induction. Averages ± SD for triplicate samples are shown. P-values are shown in the figure.

Figure 4. C-di-GMP as a universal biomarker for PAO1/p_{BAD}-yjhH in vitro dispersed cells with induction by L-arabinose in comparison with planktonic cells [AU: Edit correct?]. Relative quantification of c-di-GMP measured by peak area on HPLC normalized by protein concentration (Step 35). The dispersed cells possessed significantly lower levels of c-di-GMP than the planktonic cells. Averages ± SDs for triplicate samples are shown. P-values are shown in the figure.

Figure 5. Biomarkers specific to *P. aeruginosa* in vitro dispersed cells post biofilm dispersal in comparison with planktonic cells [AU: Edit correct?]. Fluorescence intensity of (a) p_{cdrA}-gfp expression in PAO1/p_{BAD}-yjhH (planktonic or 0.25% arabinose-dispersed) cultures, as measured

from microscopy images by ImageJ software (Step 38). (b) Relative fluorescent unit of p_{pvdA} - gfp of PAO1/ p_{BAD} - $yhjH$ (planktonic or 0.25% arabinose- dispersed) cultures after 5-h treatment, as measured by Tecan microplate reader (Step 41). Lower expression of GFP for both transcriptional fusions were observed in dispersed cells, compared to planktonic cells. Averages $\pm$ SD for triplicate samples are shown. P-values are shown in the figure.

Figure 6. Pyoverdine production of dispersed cells and planktonic cells measured in PAO1/ p_{BAD} - $yhjH$ (planktonic or 0.25% arabinose-dispersed) in vitro cultures by means of fluorescence (Ex400 nm/Em460 nm) by the Tecan microplate reader (Step 44). Dispersed cells produced significantly lower levels of pyoverdine than planktonic cells. Averages $\pm$ SD for triplicate samples are shown. P-values are shown in the figure.

Figure 7. *In vivo* dispersal of *P. aeruginosa* biofilms. Mice had silicone implants inserted in the peritoneal cavity, which were pre-colonized with either PAO1/ p_{BAD} - $yhjH$ (open circles) or PAO1/pJN105 (filled circles). The mice received intraperitoneal injections of 2% L-arabinose at 24 and 26.5 h post-insertion. The mice were euthanized at 29 h, the implants were removed, and the CFU per implant was determined (filled and open triangles, Step 79). The peritoneal cavity was hereafter flushed with 0.9% NaCl, and the CFU ml⁻¹ was determined (filled and open squares, Step 79). Finally, the spleens were removed, and the CFU per spleen was determined (filled and open diamonds, Step 79). Lines represent medians, n represents sample number and P-values are shown in the figure (Step 80). The animal experiments were performed in accordance to the European Convention and the Directive for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and the Danish law on animal experimentation, with the authorization and approval from the National Animal Ethics Committee of Denmark (The Animal Experiments Inspectorate, dyreforsogstilsynet.dk) under

the permit number 2010/561-1817. At 0 h, both PAO1/p_{BAD}-yjhH and PAO1/pJN105 colonized on the implant at equivalent CFU ml⁻¹. With the addition of 2% L-arabinose to disperse PAO1/p_{BAD}-yjhH biofilm, the CFU ml⁻¹ on the implant decreased significantly more than PAO1/pJN105. Consequently, the dispersed cells entered into the peritoneal cavity and spleen, which resulted in higher CFU ml⁻¹ for PAO1/p_{BAD}-yjhH than PAO1/pJN105.

Author contributions statements

S.L.C., M.Y., T. E. R. and M.R. performed the *in vitro* dispersal experiments. L. D. C. performed the *in vivo* dispersal experiments. S.L.C., M.G., T.T.N. and L.Y. wrote the manuscript. M.G., T.T.N. and L.Y. designed the experiments.

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Competing financial interests

The authors declare that they have no competing financial interests.

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