Cyclic-di-GMP signaling controls metabolic activity in Pseudomonas aeruginosa

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**Highlights**

- The switch from planktonic to biofilm growth is associated with high metabolic rates.
- Induction of biofilm via c-di-GMP signaling increases metabolic expenditure.
- High metabolic rates are mainly caused by production of biofilm matrix components.

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**In brief**

Lichtenberg et al. investigate the metabolic rates of bacteria living planktonically and in biofilms. They show that the phenotypic switch, which is regulated by c-di-GMP signaling, is associated with increased metabolic rates, which are mainly caused by production of biofilm matrix components.
Cyclic-di-GMP signaling controls metabolic activity in *Pseudomonas aeruginosa*

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SUMMARY

Bacteria in biofilms are embedded in extracellular matrix and display low metabolic activity, partly due to insufficient diffusive exchange of metabolic substrate. The extracellular matrix and low metabolic activity both contribute to the high antibiotic tolerance—the hallmark of biofilm bacteria. The second messenger molecule, c-di-GMP, regulates biofilm development in *Pseudomonas aeruginosa*, where high internal levels lead to biofilm formation and low levels are associated with planktonic bacteria. Using a microcalorimetric approach, we show that c-di-GMP signaling is a major determinant of the metabolic activity of *P. aeruginosa*, both in planktonic culture and in two biofilm models. The high c-di-GMP content of biofilm bacteria forces them to rapidly spend a large amount of energy on the production of exopolysaccharides, resulting in a subsequent low metabolic state. This suggests that the low metabolic state of bacteria in mature biofilms, to some extent, is a consequence of a c-di-GMP-regulated survival strategy.

INTRODUCTION

Bacteria residing in biofilms are thought to be phenotypically distinct from their planktonic counterparts. Several publications have identified specific biomarkers to characterize this distinction, such as differential gene expression (Folsom et al., 2010), secretion of extracellular polymers (Costerton et al., 1995; Goltermann and Tolker-Nielsen, 2017), virulence factors (Hauser, 2011), metabolism (Solokhina et al., 2017), and increased tolerance toward antibiotics and host responses (Bjarnsholt et al., 2013; Ciofu and Tolker-Nielsen, 2019). However, physical aggregation and increased antibiotic tolerance are the most dominant and consistent of these biofilm-associated phenotypes. Traditionally, biofilm research has focused on surface-attached biofilms, but there is an increasing focus on embedded and non-attached biofilm aggregates (Alhede et al., 2011; Kragh et al., 2016; Secor et al., 2018). In the majority of biofilm-related infections, bacteria are found as non-attached aggregates embedded in the host material, such as slough and mucus (Bjarnsholt et al., 2013). The difference in growth geometry between planktonic bacteria and surface-attached and non-attached biofilms may result in distinct microenvironments (Stewart and Franklin, 2008; Stewart et al., 2016, 2019), which can differentially impact the metabolic activity and number of bacteria aggregating (Sønderholm et al., 2017).

The increased tolerance of biofilms toward antibiotics is frequently attributed to a lowered metabolic activity of the bacteria (Kolpen et al., 2017; Lopatkin et al., 2019). Thus, slow metabolism is one of the hallmarks in both in vivo and in vitro biofilms (Kolpen et al., 2022), although other factors such as upregulated efflux pumps (Bartell et al., 2019; Frimodt-Møller et al., 2018; Pamp et al., 2008), protection by matrix components (Cao et al., 2016; Chiang et al., 2013; Goltermann and Tolker-Nielsen, 2017; Tseng et al., 2013), and SOS responses (Bernier et al., 2013; Nguyen et al., 2011) can also be of importance.

The transition from the planktonic phenotype to the sedentary biofilm phenotype is regulated by the central messenger molecule cyclic diguanylate (c-di-GMP) in various bacteria (Hengge, 2009), including *Pseudomonas aeruginosa* (Hickman et al., 2005; Kuchma et al., 2007). C-di-GMP positively regulates the production of biofilm matrix components, including alginate, pel and psl polysaccharides, CdrA adhesin, and Cup fimbriae in *P. aeruginosa* (Baraquet and Harwood, 2013; Borlee et al., 2010; Fazli et al., 2014; Hickman et al., 2005; Kuchma et al., 2007; Rao et al., 2008). However, the impact of c-di-GMP signaling on the metabolism of bacteria in biofilms is not known.

Non-invasive measurements of metabolic activity in biofilms are not trivial, as many current methods rely on disrupting the biofilm and altering the chemical microenvironment, though some genetic (Kragh et al., 2014; Poulsen et al., 1993; Stemberg et al., 1999) and chemical reporters for metabolic activity do exist (Corte et al., 2019). However, most of these methods only cover part of the metabolic output linked to, e.g., growth or production of molecules produced under certain conditions (Poulsen et al., 2010), both in planktonic culture and in two biofilm models. The high c-di-GMP content of biofilm bacteria forces them to rapidly spend a large amount of energy on the production of exopolysaccharides, resulting in a subsequent low metabolic state. This suggests that the low metabolic state of bacteria in mature biofilms, to some extent, is a consequence of a c-di-GMP-regulated survival strategy.
et al., 1993; Whooley and McLoughlin, 1982). Since metabolic activity seems to play a distinctive role between bacteria in planktonic and biofilm states, a fine-scale measurement of energy release could be a biomarker of interest.

Microcalorimetry has been used in material research for decades but is now gaining interest in life sciences, where it has been used to discriminate bacteria, assess treatments, and estimate minimum inhibitory concentrations (MICs) of antibiotics (Baldoni et al., 2009; Braissant et al., 2015; Butini et al., 2019; Solokhina et al., 2017; Tellapragda et al., 2020). This technique measures energy release as heat flow, providing a real-time measurement of the population-level metabolic activity in a sample (Braissant et al., 2015, 2020; Wadsø et al., 2017).

In the present study, we used non-invasive, isothermal microcalorimetry to examine the metabolic activity of P. aeruginosa bacteria in surface-attached biofilms (SBs), non-attached biofilm aggregates embedded in alginate beads (ABs), and planktonic cultures (PC). Subsequently, we investigated the impact of c-di-GMP signaling and the production of biofilm matrix components on the metabolic rate in both planktonic and biofilm bacteria.

RESULTS

Metabolic activity of ABs, SBs, and PCs
The physiology of bacteria in biofilms is often assumed to be different from PCs due to a stratification of resources, but the precise metabolic behavior is unclear. Many studies quantify metabolism by growth assays, which may not reflect the turnover of energy. Therefore, we aimed to characterize the metabolic energy turnover of P. aeruginosa by isothermal microcalorimetry. To ensure that thermograms reflected metabolic activity, we treated with 10 μg mL⁻¹ tobramycin. Tobramycin prevents mRNA translation, and upon treatment, the heat flow decreased immediately and remained near the baseline throughout the experiment (Figure S1).

We grew P. aeruginosa either as planktonic shaken culture, in ABs, where embedded biofilm aggregates develop over time (Sønderholm et al., 2017, 2018), or in the bottom of plastic wells yielding a flat attached biofilm (Kragh et al., 2019). Untreated P. aeruginosa PAO1 preincubated in all three models (AB, PC, and SB) showed immediate signal increases. Thermograms from all models showed 2 peaks in heat flow, but the position and magnitude of the peaks varied between models (Figures 1A–1C). The recorded thermograms displayed some variation in temporal dynamics as well as in the magnitude of the heat flow. Generally, the peak metabolic activity of the surface biofilm grown bacteria was lower than that of the PC and ABs (p < 0.05). Higher bacterial concentration decreased the time to peak metabolic activity, as shown for other microbes previously (Braissant et al., 2015; Wadsø et al., 2017) (Figure S2A).

However, the shape of the thermograms did not seem to change as a function of the bacterial concentration and principal-component analysis (PCA) showed that most of the variance could be explained by the model and only to a small degree by the bacterial concentration (Figure S2B).

Therefore, we performed PCA (with no scaling) on the raw thermograms to assess whether P. aeruginosa grown in the different models showed distinct clustering. A clear separation across the two first principal components (Figure 1D) was found for the different models but not for the preincubation time. The number of viable bacteria in each well was not significantly different between models or preincubation times at the end of experiments (p > 0.05; Tukey’s multiple comparison test), but the 24 and 48 h preincubated ABs contained more bacteria than the other models at the start of the experiment (p < 0.01; Tukey’s multiple comparison test) (Figure S3).

C-di-GMP alters the metabolic activity of bacteria in biofilms and PC
To evaluate the role of the second messenger c-di-GMP on the metabolic activity, we employed the wild-type PAO1 and engineered PAO1 strains containing either a pBAD-PA2133 fusion (Andersen et al., 2021) or a pBAD-PA1120 fusion (Rybtke et al., 2012). pBAD functions as an arabinose-inducible promoter in P. aeruginosa (Newman and Fuqua, 1999), and throughout this study, we used arabinose to induce strong expression of the pBAD-PA2133 and pBAD-PA1120 genes. The PA2133 gene encodes a phosphodiesterase that degrades c-di-GMP, resulting in a strongly reduced c-di-GMP content in the strain with the pBAD-PA2133 fusion (Andersen et al., 2021). Conversely, PA1120 encodes a diguanylate cyclase that synthesizes c-di-GMP, resulting in a highly increased level of c-di-GMP in the strain with the pBAD-PA1120 fusion (Rybtke et al., 2012).

In PC and the two biofilm models, the metabolic peak of the c-di-GMP overproducer was significantly higher than that of the wild-type PAO1 (p < 0.001; Figures 2A–2C). Conversely, the metabolic peak of the c-di-GMP-depleted strain was significantly lower than that of the PAO1 wild type when the strains were grown as embedded biofilms (p < 0.001; Figure 2A). However, when the c-di-GMP-depleted strain was grown in the SB and PC models, the metabolic peaks were not significantly different from those of the wild type (p > 0.05; Figures 2B and 2C). It should be noted that the SB model has the inherent weakness that it also contains planktonic cells, which may result in less clear differences when comparing thermograms between this model and the bacteria grown in PC. In particular, the c-di-GMP-depleted strain formed only a small amount of SBs (data not shown). Therefore, most of the c-di-GMP-depleted bacteria were planktonic in the SB model. In general, however, high bacterial levels of c-di-GMP resulted in rapid usage of the available energy, followed by a subsequent low metabolic activity (Figure 2). The large difference between the thermograms of the c-di-GMP overproducer and the c-di-GMP-depleted strain suggests that c-di-GMP-dependent processes govern the metabolism of biofilm bacteria to a large extent.

PCA showed, as expected, that the PAO1 wild type clustered in three separate clusters according to the growth model. When the c-di-GMP overproducer was grown as SB, it clustered distinctly from all other strains and models (Figure 2D). However, the c-di-GMP overproducer grown as planktonic bacteria and in embedded biofilms clustered together but separately from any other strain or model. The c-di-GMP-depleted strain clustered together with the planktonic wild type, independently of which growth model was used (Figure 2D).

To compare and visualize the mutant strains with their wild-type parent strain, a logistic classifier was trained on the PAO1
wild-type data and then subsequently applied to the mutant data. We can, thus, obtain an effective description of a mutant strain in various conditions as a superposition of wild-type behavior. Figure 3 shows the result of this analysis in a ternary plot, where each axis indicates how much of the observed metabolic behavior can be described as similar to the wild type grown as planktonic, surface biofilm, or alginate biofilm, respectively.

As indicated from the PCA, the thermograms of the c-di-GMP-depleted strain grown as planktonic bacteria were 99.4% similar to planktonic PAO1. In contrast, the planktonic c-di-GMP overproducer resembled the growth of PAO1 in ABs (47.6%) and as planktonic bacteria (45.9%). In addition, the surface biofilms of the c-di-GMP overproducer were 81.5% similar to PAO1 surface biofilms, while the surface biofilms of the c-di-GMP-depleted strain more closely resembled planktonic PAO1 (86.5%) and were only 6.3% similar to surface-attached PAO1. A similar pattern was found for ABs where the thermograms of the c-di-GMP-depleted strain were 10.4% related to PAO1 in ABs. In comparison, the c-di-GMP overproducer in ABs closely resembled the thermograms of PAO1 in ABs (89.4%) (Figure 3).

**c-di-GMP-regulated exopolysaccharide production is a major determinant of metabolic activity**

To investigate the relationship between c-di-GMP-regulated exopolysaccharide production and the metabolic activity, we created exopolysaccharide-deficient strains with high or low content of c-di-GMP. The strains had deleted algD, pelA, and pslBCD genes, rendering them unable to produce the polysaccharides alginate, Pel, and Psl (hereafter referred to as exopolysaccharide deficient) (Goltermann and Tolker-Nielsen, 2017; Rybtke et al., 2020). In addition, they contained either pBAD-PA1120 or pBAD-PA2133, resulting in either high or low c-di-GMP content. The thermograms of these strains were compared with those of the PAO1 wild type and the c-di-GMP overproducer strain with intact exopolysaccharide synthesis (Figure 4). The exopolysaccharide-deficient strains form no, or very little, SBs (data not shown), and therefore we only grew these strains as alginate-embedded biofilms and as PCs. In both models, the peak metabolism was significantly different between the strains (Figure S4). The c-di-GMP overproducer showed the highest peak, then followed by the PAO1 wild
type, the exopolysaccharide-deficient c-di-GMP overproducer, and finally the exopolysaccharide-deficient c-di-GMP-depleted strain (Figures 4A and 4B). The slightly increased metabolic activity of the exopolysaccharide-deficient c-di-GMP overproducer compared with the exopolysaccharide-deficient c-di-GMP-depleted strain is likely due to the former strain's production of high levels of CdrA and Cup (Vallet et al., 2001; Borlee et al., 2010).

PCA showed that the c-di-GMP overproducer with intact exopolysaccharide production grown in ABs and as PC clustered together while clustering separately from the wild type, as seen in the previous experiments (Figure 4C). The exopolysaccharide-deficient c-di-GMP overproducer clustered distantly from the c-di-GMP overproducer with intact exopolysaccharide production and much closer to the exopolysaccharide-deficient c-di-GMP-depleted strain. Apparently, the growth model did not seem to influence the thermograms of these strains. Together, the results indicate that the high and fast energy expenditure of biofilm bacteria with high c-di-GMP content, to a large extent, is caused by the synthesis of biofilm matrix components.

We investigated this hypothesis further using *P. aeruginosa* strains that are engineered with inducible expression of a single exopolysaccharide, as well as deletions of the other exopolysaccharide genes. Specifically, we used pBAD-pelDpslBCD∆algD and pBAD-pslDpelA∆algD strains (Rybtke et al., 2020) where the production of Pel or Psl, respectively, can be induced with arabinose. Arabinose induction led to significantly higher peak metabolic activities in the arabinose-induced pBAD-pelDpslBCD∆algD and pBAD-pslDpelA∆algD strains compared with uninduced strains (p < 0.0001; Figure 5A). Concomitant measurements of growth showed that induction of Pel and Psl...
Here, we show that the metabolic trajectory of *P. aeruginosa* in biofilms differs from that of bacteria during planktonic growth. As c-di-GMP is widely recognized as regulating planktonic growth, with high levels of c-di-GMP associated with biofilm formation, we asked whether the level of c-di-GMP had an influence on the metabolism of the bacteria. Overproduction of c-di-GMP led to distinct metabolic activity profiles regardless of the growth model. When grown as SBs, the thermograms of the c-di-GMP overproducer mostly resembled that of wild-type SBs. Still, it represented the most distinct metabolic activity profile (Figure 2D). The metabolism of the c-di-GMP overproducer strain grown either planktonic or as embedded biofilm resembled that of the embedded wild-type biofilm (Figure 2). Common for the strain with high c-di-GMP production across the models was a higher and less prolonged expenditure of energy (Figure 2). Thus, the high content of c-di-GMP in biofilm bacteria appears to promote a rapid usage of energy followed by low metabolic activity.

Conversely, peak metabolism was lower in strains with low c-di-GMP levels, and metabolic activity continued for longer. The metabolism of the c-di-GMP-depleted strain resembled that of wild-type bacteria during planktonic growth regardless of the model it was grown in. Even in ABs where the bacteria are forced to grow in high-density microcolonies and have approximately the same microenvironmental conditions as the wild-type strain, the metabolic activity profile of the c-di-GMP-depleted strain was much closer to that of a planktonic wild-type culture than to that of the wild-type biofilm aggregates (Figure 3).

Subsequent experiments employing exopolysaccharide-deficient *P. aeruginosa* strains with either high or low intracellular content of c-di-GMP indicated that the fast energy usage of bacteria with high c-di-GMP content primarily is due to the synthesis of biofilm matrix components. Our results suggest that the production of c-di-GMP-regulated biofilm matrix components is induced during the formation of both embedded biofilms and SBs, suggesting that it may be a general feature of aggregated growth. Interestingly, the apparent increase in energy expenditure in matrix-producing strains did not result in lower growth rates (Figure 5). Bacteria are assumed to turn over substrate as fast as possible, irrespective of what that substrate is turned into. There are, however, important exceptions to this mode. For example, a downregulation of metabolic activity is seen in cells entering the stringent response (Traxler et al., 2008) or in persister cells (Prax and Bertram, 2014). In contrast, bacteria can enter the seemingly wasteful process of “overflow metabolism,” where oxidative phosphorylation and fermentative metabolic pathways are used in concert as a response to a replete nutrient environment (Basan et al., 2015; Szenk et al., 2017).

Collectively, the results described above indicate that the level of c-di-GMP impacts the metabolism of *P. aeruginosa* and that the high c-di-GMP level in biofilm bacteria forces the bacteria to rapidly spend a high amount of energy on the production of biofilm matrix components, resulting in a subsequent low metabolic state of the bacteria in the biofilms. This suggests that the low metabolic state of the bacteria in mature biofilms, to some extent, is a consequence of a c-di-GMP-regulated survival strategy.
The total energy in each well was not different between the models and is ultimately linked to either the availability of electron acceptors or donors for respiration in the sealed well. We found total released energy similar to what was seen before (Braissant et al., 2015). We tested the cease in metabolism and found that it was related to electron-acceptor availability, rather than electron-donor availability, by supplementing with NO₃⁻/CO₃⁻, which yielded higher total energy turnover. 

*P. aeruginosa* is able to grow anaerobically with high biomass yield on NO₃⁻/CO₃⁻ (Line et al., 2014; Strohm et al., 2007) and further on arginine and pyruvate fermentation (Eschbach et al., 2004; Schreiber et al., 2006) and reduction-oxidation reactions of self-produced phenazines (Price-Whelan et al., 2007). Thus, the limiting factor for metabolic activity in our experiments seem to be similar to that of chronic *P. aeruginosa* infections in, e.g., patients with cystic fibrosis or with chronic wounds. Here, bacterial aggregates are found entrapped in the host material and surrounded by numerous polymorphonuclear leukocytes (PMNs) (Kolpen et al., 2010; Kragh et al., 2014) functioning as an oxygen sink, and the bacterial metabolism is thus often characterized by an insufficient supply of electron acceptor rather than carbon source (Jensen et al., 2017; Kragh et al., 2014).

Based on the present work, we hypothesize that c-di-GMP-regulated matrix production and subsequent low metabolic activity function as a survival strategy and enable the bacteria to persist in chronic infections despite the continuous influx of PMNs (Bjarnsholt et al., 2009). High c-di-GMP levels in biofilm bacteria induce vast matrix production, protecting against phagocytosis and antimicrobial agents. The matrix production occurs at high cost and proceeds until oxygen is limited, resulting in low metabolic activity that further protects against antimicrobial agents. We suggest that the present survival process is
similar to spore formation (Tan and Ramamurthi, 2014) of the two bacterial genera Clostridium and Bacillus, just on a multicellular scale. The transition from vegetative growth to sporulation is differentially regulated in these bacteria, but both strategies are energetically costly and lead to a subsequent low metabolic state as well as tolerance to environmental insult.

Limitations of the study
While we have uncovered that the initial switch from the planktonic to the biofilm lifestyle may be associated with increased energy expenditure, the exact timeline for this was not uncovered. Measurements in this study were performed in a closed system and may be different in open systems where resources are not limited in the same manner. Thus, for how long this behavior can be observed is not known. It will be interesting also to investigate further the flux of energy that goes into cell division and exopolysaccharide production, respectively, and whether the rate of growth is diminished at the cost of matrix production. While we did not measure any differences in growth of the induced and uninduced exopolysaccharide (EPS) strains (Figure 5), we also note that exact quantification of strains with high matrix production is inherently imprecise, as aggregated bacteria will mask enumeration both by colony-forming unit (CFU) count and by optical measurements.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- Bacterial strains used and construction of strains
- METHOD DETAILS
  - Growth conditions
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  - Colony forming units
  - Growth curves
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Principal component analysis
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111515.

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AUTHOR CONTRIBUTIONS


Figure 5. Inducible expression of single exopolymers promotes higher peak metabolic activity
(A) Peak metabolic activity of P. aeruginosa PAO1 (blue), and engineered strains with arabinose-inducible expression of Pel but deleted genes for Psl and alginate (dark green) and strains with arabinose-inducible expression of Psl but deleted genes for Pel and alginate (light green) with and without the addition of arabinose. There was a significant effect of arabinose addition on peak metabolic activity for both engineered strains (p < 0.05; two-way ANOVA with Bonferroni multiple comparison) but not for PAO1. (B) Growth of PAO1 and Pel-inducible strain with and without induction with arabinose. (C) Growth of PAO1 and Psl-inducible strain with and without induction with arabinose. Dark shades in (B) and (C) represent addition of arabinose, and light shades represent no addition of arabinose. Data are presented as means ± SEM and arise from three separate experiments.
**REFERENCES**


## STAR METHODS

### KEY RESOURCES TABLE

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Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Thomas Bjarnsholt (tbjarnsholt@sund.ku.dk).

Materials availability
Mutant strains generated for use in this study will be made available on request, but we may require a completed Materials Transfer Agreement if there is potential for commercial application.

**RESOURCE AVAILABILITY**
**Data and code availability**
- The data reported in this paper will be shared by the lead contact upon reasonable request.
- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Bacterial strains used and construction of strains**
We used a wild-type P. aeruginosa PAO1 strain obtained from the Pseudomonas Genetic Stock Center, ECU, USA (strain PAO0001) for initial microcalorimetric measurements. For microscopy, we used a PAO1 tagged with a stable green fluorescent protein (GFP) constitutively expressed by plasmid pMRP9 (Bjarnsholt et al., 2005). To evaluate the role of the second messenger c-di-GMP on the metabolic activity, we employed the wild-type PAO1 and engineered arabinoinducible PAO1 strains containing either a pBAD-PA2133 fusion or a pBAD-PA1120 fusion. The PA2133 gene encodes a phosphodiesterase that degrades c-di-GMP resulting in a strongly reduced c-di-GMP content. Conversely, PA1120 encodes a diguanylate cyclase that synthesizes c-di-GMP resulting in a highly increased level of c-di-GMP. Two additional strains were constructed for the study. Both strains were constructed in the PAO1 ΔpelAΔpslBCΔalgD EPS deficient strain (Rybtke et al., 2020). The strains were created using the previously described three-parental mating technique (Andersen et al., 2021; Rybtke et al., 2012). Briefly, the strains were created using the recipient strain PAO1 ΔpelAΔpslBCΔalgD and the helper strain E. coli pRK600, and either the E. coli pENTRminiCTX2-pBADPA1120 (Rybtke et al., 2012) or E. coli pENTRminiCTX:pBAD-PA2133 (Andersen et al., 2021) strains to insert either pBADPA1120 or pBAD-PA2133, respectively, in the chromosome of the bacteria. Lastly, we used the P. aeruginosa PAO1 derivatives pBAD-pelΔpslBCΔalgD and pBAD-pslΔpelAΔalgD (Rybtke et al., 2020) to enable arabinose-induced expression of Pel or Psl, respectively, in strains that otherwise do not synthesize exopolysaccharide. All strains and plasmids used are described in the key resources table.

**METHOD DETAILS**

**Growth conditions**
All experiments were performed at 37 °C in R2A broth (Lab M Ltd, UK) supplemented with 0.05 M Tris-HCl buffer (pH = 7.6) and 0.5% glucose (henceforth mentioned as R2A media), and for experiments with arabinose inducible strains we supplemented with 0.2% L-arabinose. Overnight (ON) cultures were started according to (Krاغh et al., 2017) in R2A media.

**Preparation of planktonic cultures, surface-attached biofilms, and alginate beads**
All samples were prepared inside plastic inserts (non-activated calWells, Symcel, Sweden). Surface-attached biofilms were created by inoculating each insert with 200 μL of culture adjusted to a final optical density (OD450) of 0.005. Inserts were then covered with parafilm and incubated for either 24 or 48 h at 37 °C, 120 rpm. This allowed biofilm to develop on the sides and bottom of the insert. Each insert was then washed with saline (0.9% NaCl) to remove planktonic biomass. After washing, 200 μL fresh R2A media was added, and the insert was positioned in the calPlate (Symcel, Sweden). The ON culture was filtered through a sterile, syringe filter (pore size = 10 μm) to remove aggregated bacteria for planktonic cultures. The filtered culture was then diluted to an OD450 of 0.005 with fresh R2A media. An aliquot of 200 μL was added to each insert and positioned in the calPlate (Symcel, Sweden).

Alginate beads containing bacteria were produced as previously described (Sønderholm et al., 2017). Alginate beads were produced by mixing seaweed alginate (2% w/v) (Protanal LF 10/60 FT; FMC Biopolymer, Norway) with an ON culture adjusted to an OD450 of 2 to a final OD450 of 0.1. Beads were formed by extrusion dropping through a 21-gauge needle placed 3 cm above the surface of a stirred 0.25 M CaCl2 solution and left to harden for 1 h, producing beads of ⌀ = 2.4 mm (Sønderholm et al., 2017). Beads were rinsed in 0.9% saline and transferred to prewarmed (37 °C) R2A media. The beads were incubated in R2A media at 100 rpm at 37 °C for either 24 or 48 h. After incubation, beads were gently rinsed in 0.9% saline to remove non-embedded cells from the bead surface. A single bead was then placed in each insert, and the insert was filled with 190 μL fresh R2A media, resulting in a final volume of 200 μL in the insert. The insert was then positioned in the calPlate (Symcel, Sweden).

**Microcalorimeter procedure**
Microcalorimetric measurements were conducted according to the manufacturer’s procedures and guidelines (Symcel, Sweden) and as previously described (Braissant et al., 2015; Wadso et al., 2017). Each plastic insert was placed inside sterile titanium cylinders with forceps. Each cylinder was sealed with a titanium lid and tightened to identical torque (40 Nm). A rack of 48 cylinders, including 32 samples and 16 references (filled with sterile media), was inserted into the microcalorimeter (calScreener, Symcel, Sweden) running the software calView 1.033. The rack was preheated in position 1 for 10 min, then moved to position 2 for 20 min before being moved into the measuring chamber. The wells were stationary during measurements, and the system was allowed to equilibrate for approximately 30 min before stable signals were recorded. Measurements of heat flow (in μW) were recorded at a rate of 1 hertz.
Colony forming units
Colony forming units (CFU) were quantified in parallel incubated samples at t0 and at the termination of the experiment. After the inserts were removed from the titanium cylinders biomass was collected by scraping with a pipette tip (Kragh et al., 2019). All liquid was then transferred from the wells to individual 1.5 mL Eppendorf tubes, as well as the alginate bead in the case of these samples. The alginate beads were dissolved in a 200 μL 0.02M/0.05M mix of citric acid and Na2CO3, respectively, followed by 10 min shaking at 1400 rpm which have been shown not to affect viability of the bacteria (Mater et al., 1995). All samples contained in eppendorf tubes were then degassed for 5 min followed by 5 min of ultra-sonication in an ultra-sound bath (230 VAC, Branson, USA) to break up aggregates. Subsequently, samples were 10-fold serial diluted and plated on LB plates (1.5% agar) and counted following incubation for 24 h at 37°C. CFU counts rely on two technical replicates in each three biological replicates.

Growth curves
The growth curves of the strains created for this study was assessed using a plate reader (Infinite F Nano+, Tecan, Switzerland). ON cultures was filtered through a 10μm pore filter and then adjusted to an OD450nm = 0.005 in R2A media with or without supplementation of 0.2% arabinose. A 96-well microtiter plate was then prepared loading the strains into individual wells. Optical density was recorded at 595 nm every 20 min for 24 h. Samples were shaken in between measurements.

QUANTIFICATION AND STATISTICAL ANALYSIS

Principal component analysis
The raw heat flow values of different conditions were combined into a single matrix and analyzed by principal component analysis using the ‘prcomp’ function in R with default settings and no scaling. A PCA plot of each samples’ position along the first two principal component vectors was generated with the ‘factoextra’ and ‘ggplot2’ R packages.

Logistic regression
A logistic regression classifier was trained on the wild-type data [input: metabolic signal, output: class (“planktonic”, “surface biofilm”, “alginate biofilm”)]. This results in three coefficients vectors a1, a2, a3, one for each class. A mutant, for which we have a metabolic signal x, was then divided into its ‘wildtype components’ by calculating a number representing class probability bi = exp(ai ⋅ x) for each class. These are then normalized into fractions as fi = bi / (b1 + b2 + b3), which can then be plotted in a ternary plot. The classifier was regularized with an L2 norm on the coefficients.

Statistics
Two-way ANOVAs and Tukey’s Multiple Comparison tests were performed in Prism (v. 9.2, GraphPad, USA). Principal component analysis and plots were made in R (v. 4.1.1). The ternary classification was made in Python (v. 3.9.1). CalScreener measurements were conducted with three-four technical replicates and three-four independent biological replicates from experiments conducted at three-four separate time points for P. aeruginosa.