

Supplementary Information

RNA-Seq reveals that *Pseudomonas aeruginosa* mounts growth medium-dependent competitive responses when sensing diffusible cues from *Burkholderia cenocepacia*

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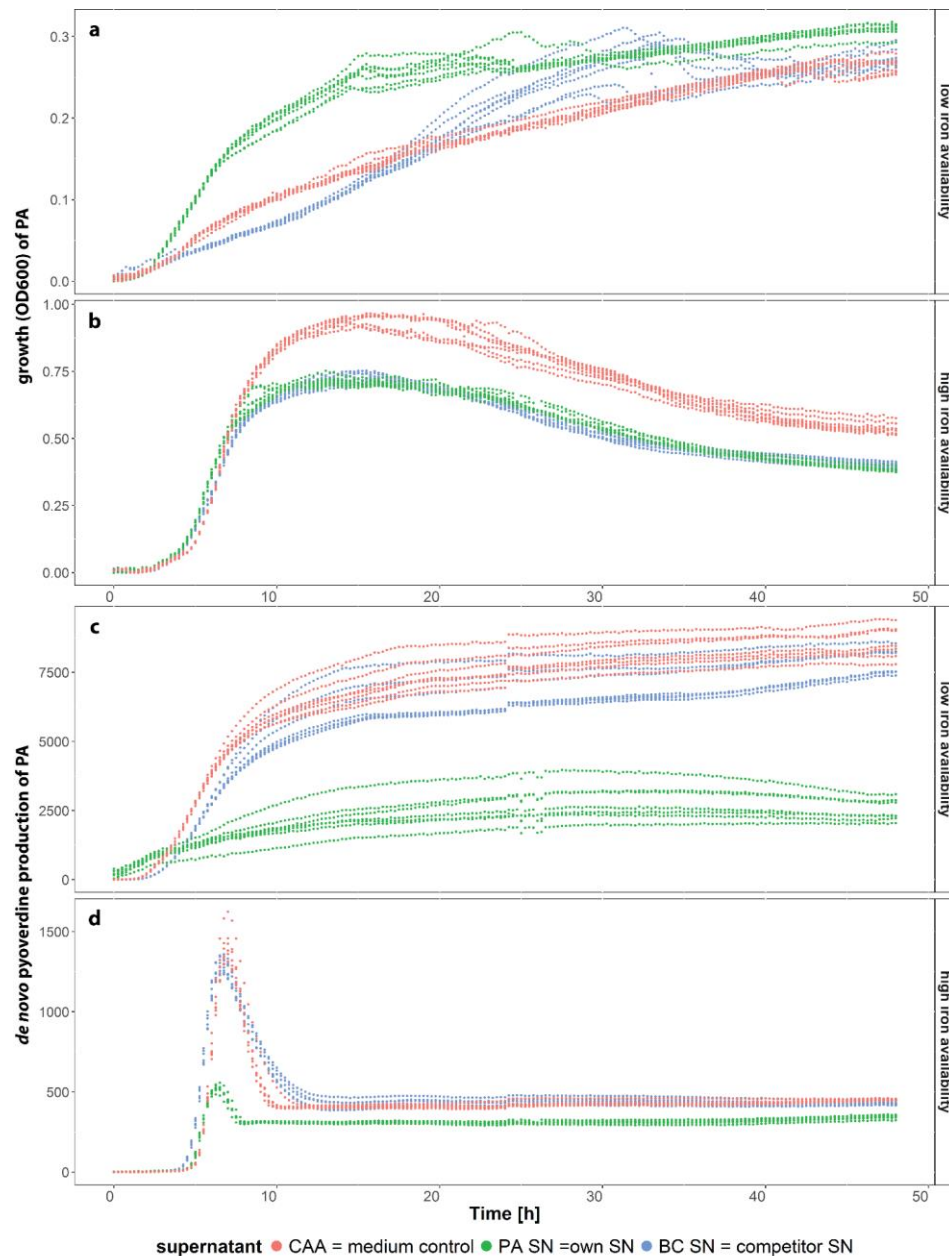
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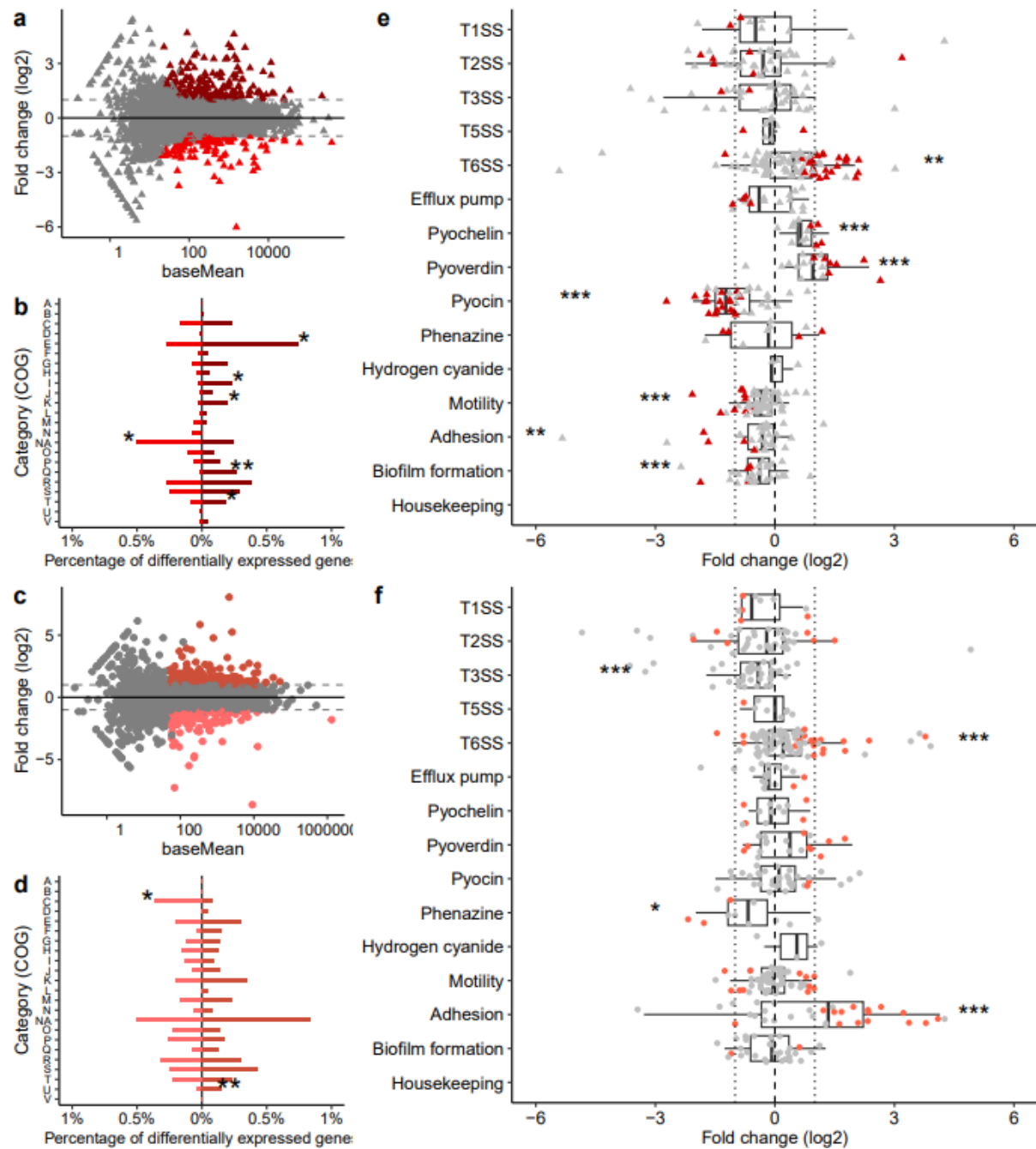
The Supplementary Information contains:

- 5 Supplementary Figures (bellow in this file).
- 13 Supplementary Data (in a separate excel file).



Supplementary Figure 1: Growth and de novo pyoverdine production of *P. aeruginosa* (PA) grown in 100% fresh CAA medium (medium control, red), in 70% fresh CAA medium mixed with 30% of its own (PA) supernatant (green) or mixed with 30% of competitor (*B. cenocepacia*, BC) supernatant (blue) at low iron availability (a) and high iron availability (b). Under iron limitation (a), PA growth is stimulated by its own supernatant due to recyclable pyoverdine present in the supernatant. In contrast, the BC supernatant and the iron-limited CAA medium do not contain such growth stimulating siderophores. The BC supernatant slightly reduces PA growth compared to the other two treatments during early growth phases. Under iron-rich conditions (b), PA and BC supernatants mainly contain spent medium and no beneficial compounds, which reduces PA's overall growth compared to the medium control. The recyclable pyoverdine from the PA supernatant leads to a lower de novo pyoverdine synthesis by

PA under iron limitation compared to the other two supernatant treatments (c). Under high iron availability, pyoverdine synthesis is low overall (d). The spent culture supernatant from PA suppresses PA's de novo pyoverdine synthesis to very low levels. Bacteria were cultured under two different iron regimes (low iron availability = CAA + 100 µg/ml transferrin; high iron availability = CAA + 20uM FeCl₃). The OD600 and the autofluorescence of pyoverdine (excitation = 400 nm, emission = 460 nm) were measured over 48 hours of 7 replicates per treatment. The slight shifts in the pyoverdine fluorescence curves after 24h represent a technical artifact. This is because the plate reader protocol allowed only to program 24-hour runs, such that the protocol had to be manually relaunched after 24 hours, which caused the shift in fluorescence readings.

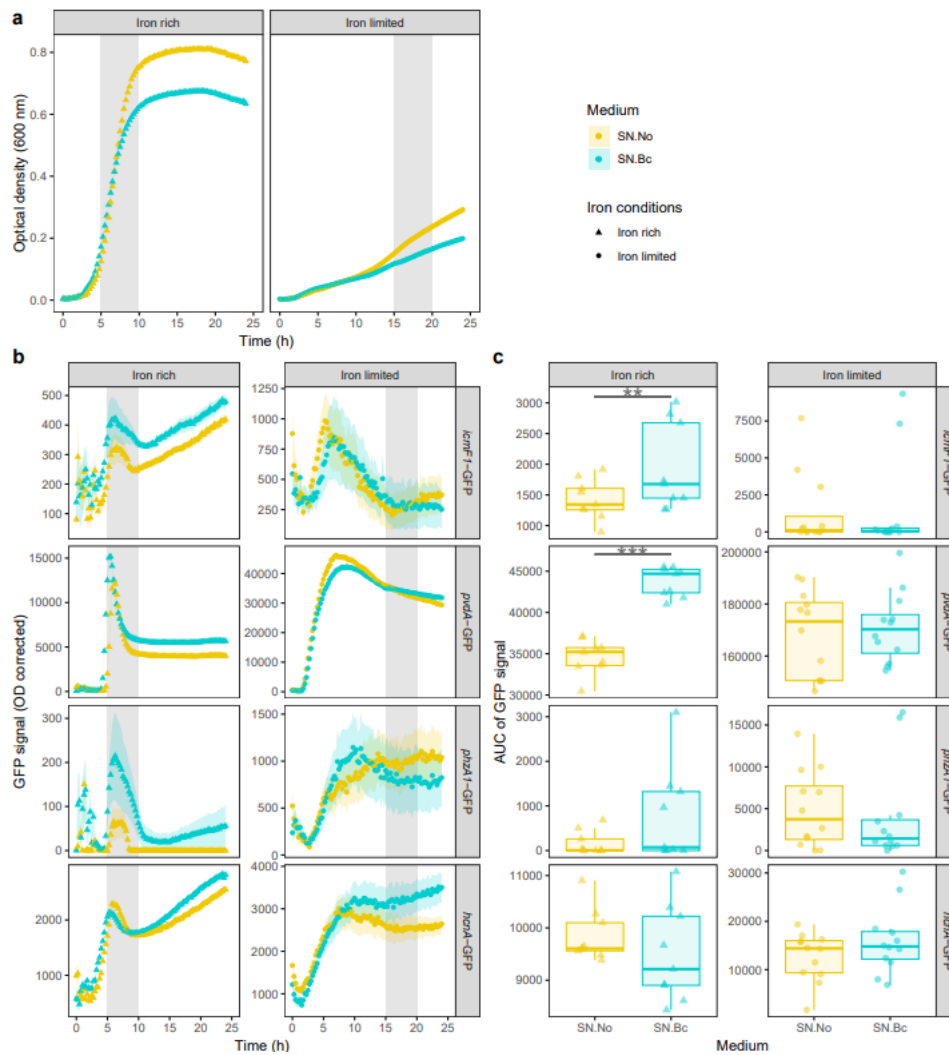


Supplementary Figure 2: Differential gene expression analysis of *P. aeruginosa* in response to its own spent supernatant. The analysis compares the gene expression of *P. aeruginosa* growing in 70% of CAA medium + 30% of its own spent supernatant (SN.Pa) with the gene expression of *P. aeruginosa* growing in fresh CAA medium (SN.No), separately for iron-rich (intense shadings and triangles – a, b, e) and iron-limited (faint shadings and circles – c, d, f) conditions.

(a + c) MA-plots depicting significantly differentially regulated genes (in color) as a function of basemean values (FDR- $p_{adj} < 0.05$ and $\log_2$ -fold change $> |1|$).

(b + d) Percentage of differentially regulated genes according to their function (COG-categories). Asterisks indicate functional classes that are significantly over- or underrepresented based on the Fisher's Exact Test for count data comparing the number of all the expressed genes with the number of the significantly up- or downregulated genes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Capital letters stand for the following COG-categories; A: RNA processing and modification ; B: Chromatin structure and dynamics ; C: Energy production and conversion ; D: Cell cycle control, cell division, chromosome partitioning ; E: Amino acid transport and metabolism ; F: Nucleotide transport and metabolism ; G: Carbohydrate transport and metabolism ; H: Coenzyme transport and metabolism ; I: Lipid transport and metabolism ; J: Translation, ribosomal structure and biogenesis ; K: Transcription ; L: Replication, recombination and repair ; M: Cell wall/membrane/envelope biogenesis ; N: Cell motility ; NA: Unassigned ; O: Posttranslational modification, protein turnover, chaperones ; P: Inorganic ion transport and metabolism ; Q: Secondary metabolites biosynthesis, transport and catabolism ; R: General function prediction only ; S: Function unknown ; T: Signal transduction mechanisms ; U: Intracellular trafficking, secretion, and vesicular transport ; V: Defense mechanisms ; W: Extracellular structures ; X: Mobilome: prophages, transposons ; Y: Nuclear structure ; Z: Cytoskeleton.

(e + f) Data show the log₂-fold change in gene expressions for gene clusters encoding competitive traits. Each dot represents an individual gene within a cluster. Colored and grey dots depict genes that are significantly and not-significantly differently regulated at the single gene level, respectively (FDR-adjusted p-values < 0.05). Asterisks indicate gene clusters that are, as a group, significantly up- and downregulated, according to a t-test conducted using zero as the expected mean (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The dotted vertical lines represent log₂-fold change = |1|.



Supplementary Figure 3: Validation experiment #1, following the expression of four genes encoding competitive traits in *P. aeruginosa* under four experimental conditions using fluorescent gene-expression reporter strains.

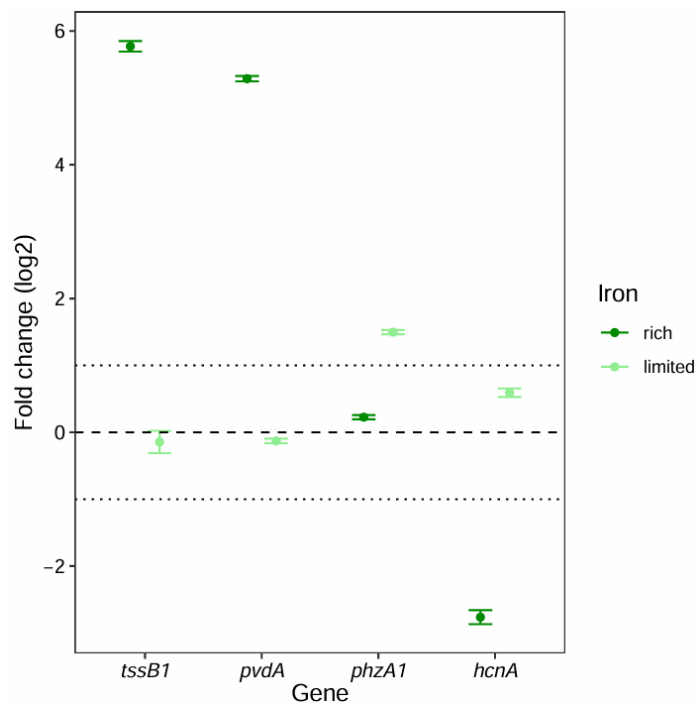
P. aeruginosa bacteria were exposed to either fresh CAA medium (SN.No, yellow) or *B. cenocepacia* supernatant (SN.Bc, cyan, 30% spent supernatant + 70% CAA). Cultures were grown for 24 hours under both iron-rich (triangles) and iron-limited (circles) conditions. We used four reporter strains to track expression of genes encoding competitive traits (*icmF1* for structural component of the T6SS, *pvdA* for pyoverdine synthesis, *phzA1* for phenazine synthesis, and *hcnA* for hydrogen cyanide synthesis). We followed growth and gene expression of all strains over time in 9-12 independent replicates. Grey shaded areas highlight the time windows during which we harvested the cells for RNA extraction for the RNA-Seq experiment: between 5 and 10 hours in iron-rich medium, and between 15 and 20 hours in iron-limited medium.

(a) Growth curves of *P. aeruginosa* under the four different culturing conditions. The data depict the mean and standard error across all four reporter strains (with 9-12 replicates per strain). Standard

errors are small and typically lay within the boundaries of the symbols. We observed that *P. aeruginosa* grows better in iron-rich compared to iron-limited conditions. In iron-rich conditions, all strains grew similarly until late exponential phase regardless of whether supernatant was supplemented or not. Towards the end of the exponential phase, *P. aeruginosa* grew to higher yield in SN.No medium because more nutrients are present compared to the SN.Bc medium. In iron-limited conditions, the growth curves of *P. aeruginosa* start to diverge between SN.No and SN.Bc at intermediate time points of the growth phase.

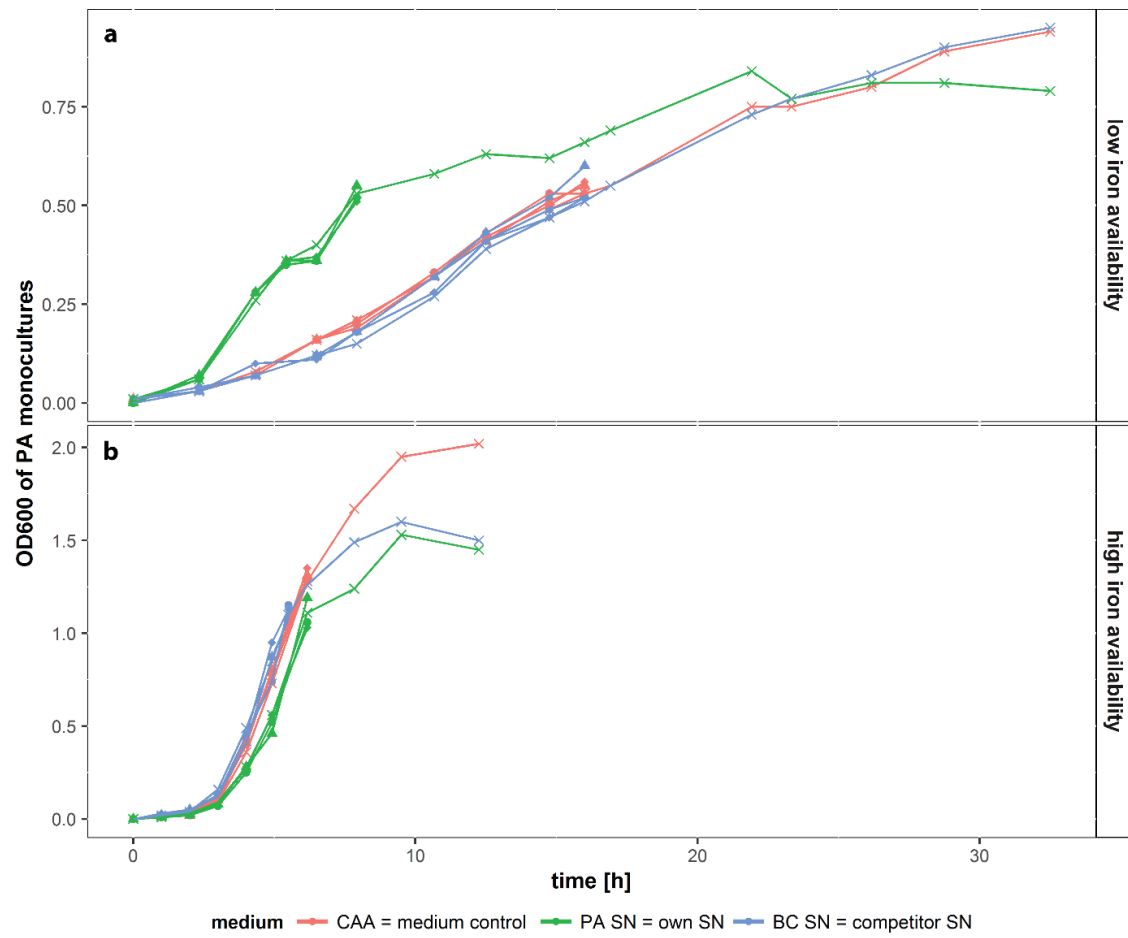
(b) Temporal gene expression trajectories of the four selected genes in the four different culturing conditions. The data depict the mean and standard error calculated across all independent replicates. All GFP values are normalized by the corresponding OD values to account for growth differences across culturing conditions. In iron-rich conditions, we observed a peak of gene expression between the 5th and 10th hour (exponential growth phase). In iron-limited conditions, gene expression rose and then stayed stable or declined during the time window of interest (15-20 hours, shaded area).

(c) Statistical comparisons of the gene expression trajectories of the four selected genes. In order to compare the GFP reporter data with the RNA-Seq transcriptomic data, we calculated the area under the curve (AUC) of the GFP signal for the time period during which samples were collected for the RNA-Seq experiment (iron-rich conditions: 5-10 hours; iron-limited conditions: 15-20 hours). Please note that a perfect comparison of time points between the GFP-reporter experiment and the RNA-Seq experiment is difficult because the culturing conditions differed (reporter experiment: 96-well plates; RNA-Seq: 1 liter glass flasks) and so statistical analysis must be interpreted with caution. Individual data points depict independent replicates, and ANOVAs were used to compare the GFP signal levels between the two culturing conditions. ** and *** respectively for $p < 0.01$ and $p < 0.001$.



Supplementary Figure 4: Validation experiment #2 quantifying the differential expression of four genes encoding competitive traits in *P. aeruginosa* by RT-qPCR.

The expression of four genes (*tssB1*, for structural component of the T6SS, *pvdA* for pyoverdine synthesis, *phzA1* for phenazine synthesis, and *hcnA* for hydrogen cyanide synthesis) was measured by RT-qPCR. Data show the log₂-fold change in gene expression by *P. aeruginosa* exposed to *B. cenocepacia* supernatant (SN.Bc) compared to fresh CAA medium (SN.No) in iron-rich (dark green) and iron-limited (light green) growth conditions. The dots and error bars respectively represent the means and the standard errors calculated using the jackknife procedure across six to nine sample pairs (arranged prior to experimentation). These results confirm our main observations from Figure 4. In response to *B. cenocepacia* supernatant exposure, *P. aeruginosa* strongly upregulates genes encoding T6SS and pyoverdine production under iron-rich conditions, while it upregulates (more moderately) genes encoding phenazines and hydrogen cyanide synthesis under iron-limited conditions.



Supplementary Figure 5: Monitoring growth of *P. aeruginosa* (PA) to find the right time point to harvest for following RNA isolation. PA grew in 100% fresh CAA medium (medium control, red), in 70% fresh CAA medium mixed with 30% of its own (PA) supernatant (green) or mixed with 30% of competitor (*B. cenocepacia*, BC) supernatant (blue). We used either low iron availability (CAA + 100 µg/ml transferrin) (a) or high iron availability (CAA + 20 µM FeCl₃) (b). PA was grown in 1 liter Erlenmeyer flasks in 200 ml medium at 37°C and 220 rpm. We followed growth by measuring the OD600 with a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences) until the cultures reached the mid- to late exponential phase, when we harvested the bacteria for following RNA isolation. We grew PA in 3 biological replicates per treatment. Each replicate of the PA and BC supernatant treatment contained a supernatant from an individually grown PA or BC monoculture under the respective iron condition. A fourth replicate was not harvested but was further monitored until the increase of OD600 levelled out, to control for the correct time point of harvesting.