

1 **Transcriptome dynamics of *Pseudomonas aeruginosa* during transition**
2 **from replication-uncoupled to -coupled growth**

3 Kathrin Alpers¹, Elisabeth Vatareck¹, Lothar Gröbe², Mathias Müsken³, Maren Scharfe⁴,
4 Susanne Häussler^{1,5,6,7*}, Jürgen Tomasch^{1,8*}

5

6 ¹Department of Molecular Bacteriology, Helmholtz Centre for Infection Research,
7 Braunschweig, Germany

8 ²Platform Flow Cytometry and Cell Sorting, Department of Experimental Immunology,
9 Helmholtz Centre for Infection Research, Braunschweig, Germany

10 ³Central Facility for Microscopy, Helmholtz Centre for Infection Research, Braunschweig,
11 Germany

12 ⁴Platform Genome Analytics, Helmholtz Centre for Infection Research, Braunschweig,
13 Germany

14 ⁵Institute for Molecular Bacteriology, Twincore, Centre for Clinical and Experimental
15 Infection Research, Hannover, Germany

16 ⁶Department of Clinical Microbiology, Copenhagen University Hospital – Rigshospitalet,
17 2100 Copenhagen, Denmark

18 ⁷Cluster of Excellence RESIST (EXC 2155), Hannover Medical School, 30265 Hannover,
19 Germany

20 ⁸Institute of Microbiology of the Czech Academy of Science, Center Algatech, Třeboň,
21 Czech Republic

22

23 *correspondence: Susanne.Haeussler@helmholtz-hzi.de

24 *correspondence: tomasch@alga.cz

25 **Abstract**

26 In bacteria, either chromosome duplication is coupled to cell division with only one
27 replication round per cell cycle or DNA is replicated faster than the cells divide thus both
28 processes are uncoupled. Here, we show that the opportunistic pathogen *Pseudomonas*
29 *aeruginosa* switches from fast uncoupled to sustained coupled growth when cultivated under
30 standard laboratory conditions. The transition was characterized by fast-paced, sequential
31 changes in transcriptional activity along the *ori-ter* axis of the chromosome reflecting
32 adaptation to the metabolic needs during both growth phases. Quorum sensing (QS) activity
33 was highest at the onset of the coupled growth phase during which only a quarter of the cells
34 keeps replicating. RNA sequencing of subpopulations of these cultures sorted based on their
35 DNA content, revealed a strong gene dosage effect as well as specific expression patterns for
36 replicating and non-replicating cells. Expression of flagella and *mexE*, involved in multi drug
37 efflux was restricted to cells that did not replicate, while those that did showed a high activity
38 of the cell division locus and recombination genes. A possible role of QS in the formation of
39 these subpopulations upon switching to coupled growth could be a subject of further research.

40

41 **Significance statement**

42 The coordination of gene expression with the cell cycle has so far been studied only in a
43 handful of bacteria, the bottleneck being the need for synchronized cultures. Here, we
44 determined replication-associated effects on transcription by comparing *Pseudomonas*
45 *aeruginosa* cultures that differ in their growth mode and number of replicating chromosomes.
46 We further show that cell cycle-specific gene regulation can be principally identified by RNA
47 sequencing of subpopulations from cultures that replicate only once per cell division and that
48 are sorted according to their DNA content. Our approach opens the possibility to study
49 asynchronously growing bacteria from a wide phylogenetic range and thereby enhance our
50 understanding of the evolution of cell-cycle control on the transcriptional level.

51

52 **Introduction**

53 Bacteria differ in the ways replication is coordinated with cell growth and division¹. In fast-
54 growing representatives, such as the model organisms *Escherichia coli* or *Bacillus subtilis*,
55 the speed of DNA replication exceeds that of cell division. This uncoupling of both processes
56 results in a gene dosage gradient along the origin(*ori*)-terminus(*ter*) axis of the chromosome.
57 The higher gene copy number closer to *ori* can be exploited to maximize expression of traits
58 needed during rapid growth and to control gene expression^{2,3}. It has been shown that moving
59 an *ori*-located *Vibrio cholerae* gene cluster coding for ribosomal proteins close to *ter* reduced
60 the growth rate of the culture, while the wild-type growth level could be restored by placing
61 two copies of this cluster at *ter*⁴. Furthermore, the timing of spore formation in *B. subtilis* is
62 an example for dosage imbalances triggering regulatory events between genes located on
63 opposite ends of the replicating chromosomes⁵. In slow-growing bacteria the chromosome is
64 duplicated only once per cell division, thus both processes are coupled⁶. In several bacterial
65 phyla, a differentiation program is triggered during this eukaryote-like cell cycle. The best
66 studied model is the bi-phasic lifestyle of *Caulobacter crescentus*. In this bacterium a
67 complex gene regulatory network precisely times the development of a flagellated from a
68 stalked cell during replication and cell division⁷.

69

70 *Pseudomonas aeruginosa* is a ubiquitous environmental bacterium, but also an opportunistic
71 pathogen frequently causing nosocomial infections of various body sites, such as the lung,
72 bloodstream, urinary tract and burn wounds⁸. Furthermore, *P. aeruginosa* poses a particular
73 threat to patients suffering from cystic fibrosis (CF)⁹. During live-long chronic infections of
74 the CF lung, the bacterium adapts and evolves towards a slow growing phenotype¹⁰.
75 Doubling times are estimated to be around 30 min under laboratory conditions in lysogeny
76 broth (LB) medium and 1.9 to 4.6 h in the CF lung¹¹. The cell cycle dynamics of *P.*
77 *aeruginosa* has been extensively studied. Its chromosome is oriented with *ori* close to the
78 center of the cell and *ter* located at the cell pole where the division plane forms. During
79 replication both *ori* move to the poles of the elongated pre-divisional cell where another
80 round of replication can be started^{12,13}. Despite the huge body of comparative transcriptome
81 data available for this important pathogen¹⁴⁻¹⁶, the effect of replication on gene expression
82 has not explicitly been studied yet.

83

84 Here we monitored growth and cell division and recorded a time-resolved transcriptome of *P.*
85 *aeruginosa* PA14 in LB medium over 10 hours at 1 hour intervals. We show that the culture
86 switches from fast replication-uncoupled to sustained coupled growth. The transition is
87 characterized by fast-paced, sequential changes in transcriptional activity along the *ori-ter*
88 axis. Furthermore, we identified replication- and non-replication-associated gene expression
89 in cells showing coupled growth using a newly developed protocol based on fluorescence-
90 activated cell sorting (FACS).

91

92 **Results**

93 **Growth and replication dynamics of *P. aeruginosa* in LB medium.**

94 In accordance with previous reports¹¹, *P. aeruginosa* cultures reached an OD₆₀₀ of 1.8±0.24
95 from a starting OD₆₀₀ of 0.05 within 4 h and a maximum doubling time of 34±1 min when
96 grown under standard laboratory conditions (Figure 1A). This exponential growth phase was
97 followed by slower growth to a maximum OD₆₀₀ of 3.17±0.11 after 9 h with an OD value
98 doubling time of 410±110 min. Cell numbers, too, increased exponentially in the first four
99 hours from 4.5*10⁷ to 7.3*10⁸±1.8*10⁸ cells/ml with a doubling time of 30±9 min, followed
100 by decreased growth to a maximum count of 3.7*10⁹±5.8*10⁷ cells/ml after 9 h with a
101 doubling time of 168±6 min. The notably slower increase of OD₆₀₀ values compared to cell
102 numbers in the last 6 h of cultivation could be explained by a decrease in cell size at later
103 growth stages that is indicative for reductive cell division (Figure 1B, Supplementary Figure
104 S1A).

105

106 The chromosome content of cells was monitored by stoichiometric staining with SYBR
107 Green (Figure 1C, Supplementary Figure S1B). In the over-night grown pre-cultures that
108 were used for inoculation, 80% of the cells contained one chromosome (C1). One hour after
109 the transfer into fresh medium already 62±7% and 30±2% of the cells contained two (C2) and
110 three chromosome equivalents, respectively, and a smaller fraction even more. This clearly
111 indicates that the cultures had moved to a phase of uncoupled growth with replication being
112 faster than cell division. After 4 to 5 h of growth, the chromosome content shifted back and
113 two distinct peaks for cells containing one or two chromosomes became visible again. The
114 presence of cells with a DNA content between the major peaks indicates actively replicating
115 cells (R). The proportion of C2 and R cells was only slowly reduced from 38±3% to 23±2%
116 between 6 h and 10 h of growth. The presence of replicating cells after 10 h of cultivation

117 was also visible on electron micrographs (Figure 1D). Our data strongly suggest that *P.*
118 *aeruginosa* shifts from fast uncoupled to sustained coupled growth cells during the course of
119 cultivation in LB medium with a short transition phase in between (Figure 1C). If the
120 doubling time of the coupled growing culture is converted to the approximately 25% of cells
121 that actually replicate, the individual division times are around 42 min, thus only slightly
122 lower than in the uncoupled growth phase.

123

124 **Transcriptome dynamics of *P. aeruginosa* during different growth phases.**

125 We monitored transcriptional changes for the full growth period in one-hour intervals. Two
126 independent experiments with two and three replicates each were carried out. The
127 transcriptomes clustered according to the growth phases except for the 6 h samples. For these
128 samples, the transcriptomes of the first experiment were closer to the transition phase, while
129 the transcriptomes of the second experiment were closer to the coupled growth phase samples
130 (Supplementary Figure S2A). The 1736 genes, which showed a significant differential
131 expression during the course of cultivation, could be assigned to eight clusters
132 (Supplementary Figure S2B and C and Supplementary Table S1).

133

134 The transition between growth-phases was characterized by fast-paced waves of transient
135 transcriptional activity (Figure 2A). Genes in clusters 1 to 3 showed a comparable high
136 expression during the first 2 h of uncoupled growth, but with a different timing of maximum
137 expression and the decline afterwards. During this growth phase, in particular transcription
138 and translation-related processes were expressed (Figure 2B), including biosynthesis of
139 tRNAs, RNA polymerase and ribosomes as well as chaperones. A high activity was seen for
140 oxidative phosphorylation and also for biosynthesis of the vitamins folate (B9) and cobalamin
141 (B12), in accordance with their respective roles in DNA and methionine synthesis.

142 Expression of the type III secretion system gene clusters *psc* and *pcr* and the *exoT* effector¹⁷
143 peaked at 2 h of cultivation followed by a steep decline.

144

145 Cluster 4 to 6 contained genes that were transiently activated at the end of exponential
146 growth. The high number of sugar and amino acid transporters as well as genes of the
147 pyruvate metabolism indicated a shift in the metabolic preferences. In particular transporters
148 for branched-chain amino acids were found to be upregulated in this transition phase, in
149 accordance with their late utilization as a carbon source observed before¹⁰. Activation of the
150 urea cycle and denitrification, and the glycogen metabolism pathway indicate changes of

151 nitrogen and carbon utilization at this stage. Cluster 7 harbored genes, which were activated
152 late in the transition phase and exhibited a stable expression throughout the coupled growth
153 phase. Denitrification genes were among them as well as genes encoding subunits of a sulfate
154 transporter and the MexHIG antibiotic efflux pump¹⁸. Finally, expression of the late
155 responding genes in cluster 8 increased between 5 and 8 h before reaching a stable level. In
156 particular, activation of the pyoverdine biosynthesis machinery, the heme acquisition protein
157 HasA and the sulfonate transport and metabolism pathway indicate a response to iron and
158 sulfur limitation in the medium, respectively.

159

160 The three components of the quorum sensing system showed different activation dynamics
161 consistent with previous data^{19,20}. While the primary QS activator *lasR/rsaL* pair was not
162 among the significantly regulated genes, its expression showed a small but consistent
163 gradually increase from 2 h cultivation on (Supplementary Table S1). The *pqsABCDE*-operon
164 was transiently activated with a peak between 4 and 5 h followed by a decline and therefore
165 found in cluster 5. The pyocyanin biosynthesis gene clusters showed the same pattern, but
166 with a much more pronounced peak. The chemotaxis operon was also activated transiently
167 during in the transition phase (cluster 6), while flagella genes were not differentially
168 regulated. The QS regulator RhlR was assigned to cluster 7 with an activation delay but
169 stable expression throughout coupled growth. The QS target genes coding for alkaline
170 protease, cyanide production and lectin B were found in the same cluster.

171

172 **Influence of gene dosage on the transcriptome during uncoupled growth.**

173 Next, we analyzed the distribution of genes in the determined clusters along the *ori-ter*-axis
174 of the chromosome. Genes active during uncoupled growth (clusters 1 to 3) were
175 predominantly located close to *ori* while those that were activated during the transition phase
176 (clusters 4 and 5) were more equally distributed along the chromosome. Genes in cluster 6,
177 activated at the end of the transition phase, already showed a tendency towards *ter*, a trend
178 that became even more pronounced for the genes in clusters 7 and 8 that increased expression
179 during coupled growth (Figure 3A). Furthermore, the average expression levels of genes in
180 the *ori*-proximal half exceeded those in the *ter*-proximal half of the chromosome during the
181 first three hours of uncoupled growth. At later time-points a balanced expression of both
182 halves of the chromosome was observed (Figure 3B). These data are in accordance with the
183 predicted gene dosage effect in cells with high replication rates.

184

185 The gene dosage effect became also visible when a general additive model was fitted to the
186 \log_2 fold-change transcriptome data along the chromosome in order to identify local trends in
187 expression dynamics that go beyond the regulation of single genes or operons. When
188 comparing subsequent time-points, with a gradual change in chromosome content, a slightly
189 lower expression was found around the terminus when transcriptomes from 3 h versus 4 h
190 were compared (and to a lesser extent for 2 h vs. 3 h, Supplementary Figure S3). This
191 comparison marks the beginning of the transition from uncoupled to coupled growth and also
192 showed the strongest shift in chromosome content during cultivation.

193

194 The position-specific differences in gene expression became more pronounced when we
195 compared transcriptomes of time-points with a higher difference in chromosome content
196 (Figure 3C). A clearly lower transcription of genes in the region surrounding the terminus of
197 replication was visible when the different growth phases were compared, in particular seen
198 for uncoupled versus coupled growth. To a lesser extent this trend was also seen for the
199 comparison of uncoupled growth to transition and transition to coupled growth phase. In
200 accordance with the analysis above, this specific reduction of gene expression proximal to,
201 and also increasing towards *ter*, can be parsimoniously explained by a change in mRNA
202 composition as a result of a higher transcriptional activity of *ori*-proximal genes, thus a gene-
203 dosage effect (indicated by the orange line in Figure 3C).

204

205 **Replication-associated transcriptome changes during coupled growth.**

206 The coupled growth with only one replication per cell division in the last 6 hours of
207 cultivation should allow to discriminate the transcriptomes of non-replicating, replicating and
208 pre-divisional *P. aeruginosa* cells. To this end, we developed a protocol employing FACS to
209 separate cells based on their chromosome content (Supplementary Text S1). In order to
210 determine the influence of fixation with formaldehyde (FA), and FACS on RNA
211 composition, we compared samples obtained during different steps of the protocol to a
212 sample fixed with RNApotect (RP) (Figure 4A). Across the three replicates, the different
213 samples showed a consistently high correlation (Figure 4B, Supplementary Figure 4A). We
214 only found 15 genes as well as the chromosomal region of 32 phage-related genes, which
215 were higher expressed in the RP- than in the FA-treated samples (Supplementary Table S2).
216 Only two genes found to be regulated during the cell cycle were also influenced by the
217 fixation method, thus rendering the protocol suitable for the intended purpose.

218

219 Next we compared the transcriptomes of the cell populations with one (C1) or two (C2)
220 chromosomes and those replicating (R). The R and C2 fractions differed from the C1
221 fraction, but were highly similar to each other (Figure 4C). Only eleven genes were found to
222 be differentially expressed exclusively when these two fractions were compared. This
223 included the *gnyDBHAL* gene cluster coding for enzymes of the acyclic isoprenoid
224 degradation pathway²¹, which showed the strongest downregulation in the R versus C2
225 fraction. The *nrdAB* genes coding for both subunits of the ribonucleotide-diphosphate
226 reductase were downregulated in the C2 fraction compared to C1 and R. This enzyme
227 catalyzes the last step in the formation of deoxyribonucleotides. In *E. coli*, its activity has
228 been linked to controlling the rate of DNA synthesis²². Furthermore, it has been shown that
229 gene expression peaks at initiation and declines towards the end of replication which is in
230 accordance with our data for *P. aeruginosa*.

231
232 Between the actively replicating R and the C1 fraction, a clear dosage effect was visible with
233 gene expression decreasing from *ori* to *ter* (Figure 4D). The same was seen for the
234 comparison of R and C2, but not when the fractions with only completely replicated
235 chromosomes, C1 and C2 were compared (Supplementary Figure S4B). The differential
236 expression of several chromosomal loci exceeded this trend dependent on the chromosomal
237 position. In the R (and C2) fraction, the genes encoding the divisome showed the strongest
238 activation compared to C1. These comprise of the *mur* and *mra* operons, encoding the
239 enzymes for remodeling the peptidoglycan layer at the division plane and the *fts* genes,
240 encoding the components responsible for septum formation²³. The recombination genes *lexA*
241 and *recG* were upregulated, too. Of note was also the transcriptional activation of one
242 genomic island, the region of genomic plasticity RGP41²⁴, consisting of only uncharacterized
243 genes. In the C1 fraction, the flagella gene clusters and chemotaxis operons, as well as the *glg*
244 genes encoding the enzymes of the glycogen metabolic pathway showed the strongest
245 activation compared to R and C2. Notably, the *mexE* gene, completely inactive in the other
246 fractions, also showed a more than 64-fold higher expression in the C1 population, by far the
247 strongest regulation in the dataset (Supplementary Table S2). It encodes the transmembrane
248 protein part of an efflux-transporter for norfloxacin and imipenem¹⁸.

249

250 **Discussion**

251 Here we showed that *P. aeruginosa* switches from replication-uncoupled to -coupled growth
252 when cultivated in LB medium, thus allowing to study the effect of replication on the
253 transcriptome. Hereby, the chromosomal gene order reflects the expression maxima during
254 both growth-phases with the genes important for fast uncoupled growth being located closer
255 to *ori* and the stationary phase genes located closer to *ter*. It has been demonstrated before
256 that the *E. coli* sigma 70 factor and its targets, which are mostly active in the exponential
257 phase, are located closer to *ori*, while the sigma S factor and its mostly stationary phase
258 active targets are located closer to *ter*²⁵. Thus, while the sigma factors transcriptionally
259 regulate downstream genes, regulon expression is additionally enhanced by a gene dosage
260 effect acting on the regulators and their target genes. Our data show the potential of
261 combining identification of different growth phases by flow cytometry with the comparison
262 of the respective transcriptomes. The gained knowledge could generally be used to identify
263 replication-associated effects on gene expression for the vast number of strains with existing
264 transcriptome data^{14,16,26}, and integrated into existing gene regulatory models^{15,27}. It could
265 further help to better understand chromosomal architecture and to explain gene order
266 evolution^{2,25,28,29}.

267

268 In the coupled growth phase, *P. aeruginosa* displays a distinct transcriptome between the
269 approx. 25% dividing and 75% non-dividing cells. Expression of flagella genes is restricted
270 to cells that are not replicating, while those that replicate differ mainly in the activity of a cell
271 division locus. Furthermore, we found that expression of *mexE*, involved in the expression of
272 an important antibiotic resistance trait is restricted to the non-dividing cells. This induction of
273 subpopulations during the switch in growth phases is coincidental with the activation of the
274 *rhl* QS system. Cell communication induced population heterogeneity has been shown for *P.*
275 *aeruginosa* before³⁰ and is also common in other bacteria^{31–33}. It might also be the trigger
276 switching the replication mode and restricting activity of the flagella gene clusters to the non-
277 dividing cells. In contrast to chemotaxis, flagella gene expression has not been described to
278 be controlled directly by QS before^{19,20}. However, we also did not find them differentially
279 expressed in the culture as a whole, but only in a subpopulation. Thus, a possible connection
280 between communication and development of motility in a fraction of cells might have been
281 overlooked and is worth a closer investigation. Furthermore, slow-growing QS-defective
282 mutants frequently evolve during CF infections^{34,35} It would be interesting to determine if

283 these strains reproduce by coupled growth only and how the transcriptome is affected by this
284 change.

285

286 The highly similar transcriptomes of replicating and pre-divisional cells indicate that in *P.*
287 *aeruginosa* no distinct phases of a differentiation program are coupled to progressing
288 replication. This is in stark contrast to the precisely timed cell cycle of *C. crescentus* with a
289 defined order of gene activity as cells replicate³⁶. Transcriptome dynamics during replication
290 has so far only been determined for a couple of model bacteria³⁶⁻³⁹. Key to these studies was the
291 ability to synchronize the cell cycle within the cultures. Our newly developed method based
292 on cell sorting according to DNA content allows for identification of replication-specific gene
293 expression without the need for synchronization, as long as the cells grow slowly with
294 coupled replication and cell division. Not only cell sorting, but also complementary recent
295 advances in single cell sequencing⁴⁰ open up the path to comparative analysis of larger
296 groups of bacteria, thus contributing to a better understanding of the evolution of cell-cycle
297 control at the transcriptional level⁴¹.

298

299 **Material and Methods**

300 **Strains and growth conditions.**

301 *Pseudomonas aeruginosa* PA14⁴² was grown in Lysogeny Broth (10 g/L tryptone, 5 g/L yeast
302 extract, 10 g/L NaCl) at 37°C and 160 rpm shaking. The growth of cultures inoculated to a
303 starting OD₆₀₀ of 0.05 was followed for 10h and samples for determination of OD₆₀₀, cell
304 count, DNA content, and RNAseq were withdrawn every hour. For FACS-based sorting,
305 cultures were inoculated to a starting OD₆₀₀ of 0.2 and samples were prepared after 5 h when
306 the coupled-growth mode was stably reached.

307

308 **Flow cytometric determination of cell number and chromosome content.**

309 100 µL of culture were mixed with 80 µL of 25% glutaraldehyde in H₂O and incubated for 5
310 min. 820 µL of PBS were added and a dilution series up to 1:1000 was prepared. 10 µL of
311 SYBR Green (100x) was added to 1 mL of diluted culture. After an incubation time of 20
312 min, the sample was measured on a BD FACS Canto flow cytometer (BD Biosciences,
313 Heidelberg, Germany). After gating based on centered forward and sideward scatter, cells
314 were identified and chromosome content quantified by fluorescence detection in the FITC

315 channel (excitation 488 and emission 535 nm). Data processing and analysis were performed
316 with the R package ggcryo⁴³.

317

318 **Electron microscopy.**

319 Bacteria were fixed by addition of glutaraldehyde (final concentration 2%) for 30 minutes,
320 and addition of formaldehyde (final concentration 5%) into the culture medium. EM sample
321 preparation was performed as previously described⁴⁴ with slight modifications. In brief,
322 samples were washed twice with TE-buffer and fixed to poly-l-lysine coated round cover
323 slips. After additional washing steps, the samples were dehydrated in a gradient series of
324 acetone (10%, 30%, 50%, 70%, 90%) on ice and two steps with 100% acetone at room
325 temperature (each step for 10 minutes). Afterwards, samples were critically point dried with
326 the CPD300 (Leica Microsystems, Wetzlar, Germany), mounted to aluminum pads and
327 sputter coated with gold-palladium. Images were acquired with a field emission scanning
328 electron microscope Merlin (Zeiss, Jena, Germany) equipped with an Everhart Thornley and
329 an inlens detector and operating at an acceleration voltage of 5kV.

330

331 **RNAseq library preparation from whole cultures.**

332 Depending on the density, 1 to 2 mL of culture were mixed with the same volume
333 RNAProtect™ Bacteria Reagent (Qiagen, Hilden, Germany) incubated for 10 min and
334 centrifuged. The pellets were flash-frozen and stored at -70°C. RNA extraction was carried
335 out with the RNeasy Plus Kit in combination with QIAshredder™ columns (Qiagen, Hilden,
336 Germany). Treatment with DNase I was performed in solution. Multiplexed libraries were
337 generated from directly barcoded fragmented RNA according to a previously published
338 custom protocol⁴⁵, including rRNA removal with the RiboZero Kit (Illumina, San Diego,
339 USA).

340

341 **Fluorescence-activated cell sorting for RNAseq of subpopulations.**

342 The method was developed based on a previously published study⁴⁶. A step-by-step protocol
343 for sample preparation, sorting and RNA isolation is provided in Supplementary Text S1.
344 Key to successful RNA recovery is the gentle formaldehyde fixation at 4°C. Aliquots of fixed
345 samples were adjusted to approx. 1.8×10^7 cells/mL in 30 ml volume each and stained with
346 SYBR Green. Sorting of 5.4×10^8 cells based on the FITC-signal (see above) directly into
347 RNAProtect was performed with the BD FACSAria Fusion (BD Biosciences, Heidelberg,
348 Germany). The sorted cells were collected on a filter from which RNA was extracted using a

349 combination of Lysozyme and Proteinase K digestion with bead beating, and purified with
350 NucleoZOL (Takara Bio, Göteborg, Sweden). Ribosomal RNA depletion was performed with
351 the NEBNext Bacteria kit (NEB, Frankfurt, Germany). The libraries were prepared with the
352 TruSeq kit (Illumina, San Diego USA).

353

354 **Transcriptome analysis**

355 Sequencing of all libraries was performed on a NovaSeq 6000 (Illumina, San Diego, USA) in
356 paired-end mode with 100 cycles in total. Reads were filtered with fastQC-mcf
357 (<https://github.com/ExpressionAnalysis/ea-utils>) and mapped to the *P. aeruginosa* PA14
358 genome (RefSeq accession GCF_000404265.1) using bowtie2⁴⁷. FeatureCounts was used to
359 assess the number of reads per gene⁴⁸. Normalization and identification of significantly
360 differentially regulated genes (FDR < 0.05, absolute log₂ fold change (FC) > 1) was
361 performed in R using the glmTreat-function of edgeR⁴⁹. Cluster assignment of differentially
362 expressed genes was performed with the package mfuzz⁵⁰.

363

364 **Data availability**

365 RNAseq raw data have been deposited at the NCBI gene expression omnibus database under
366 accessions GSE159698 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159698>)
367 and GSE217100 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE217100>).

368

369 **Acknowledgements**

370 S.H. was funded by the Lower Saxony Ministry for Science and Culture (Bacdata ZN3428),
371 the European Union (EU, ERC Consolidator Grant COMBAT 724290) and by the Deutsche
372 Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence
373 Strategy (EXC 2155 390874280). Furthermore, S.H. received additional funding from the
374 DFG (DFG SPP 1879) and the Novo Nordisk Foundation (NNF 18OC0033946). J.T.
375 received funding from the mobility grant of the Czech Ministry of Education
376 (CZ.02.2.69/0.0/0.0/18_053/0017705). The authors thank Astrid Dröge and Ina Schleicher
377 for technical support.

378 References

- 379 1. Reyes-Lamothe R, Sherratt DJ. The bacterial cell cycle, chromosome inheritance and cell
380 growth. *Nat Rev Microbiol.* 2019;17(8):467-478. doi:10.1038/s41579-019-0212-7
- 381 2. Couturier E, Rocha EPC. Replication-associated gene dosage effects shape the genomes
382 of fast-growing bacteria but only for transcription and translation genes. *Molecular
383 Microbiology.* 2006;59(5):1506-1518. doi:10.1111/j.1365-2958.2006.05046.x
- 384 3. Slager J, Veenig JW. Hard-Wired Control of Bacterial Processes by Chromosomal Gene
385 Location. *Trends in Microbiology.* 2016;24(10):788-800. doi:10.1016/j.tim.2016.06.003
- 386 4. Soler-Bistué A, Mondotte JA, Bland MJ, Val ME, Saleh MC, Mazel D. Genomic
387 Location of the Major Ribosomal Protein Gene Locus Determines *Vibrio cholerae* Global
388 Growth and Infectivity. *PLOS Genetics.* 2015;11(4):e1005156.
389 doi:10.1371/journal.pgen.1005156
- 390 5. Narula J, Kuchina A, Lee D yeon D, Fujita M, Süel GM, Igoshin OA. Chromosomal
391 Arrangement of Phosphorelay Genes Couples Sporulation and DNA Replication. *Cell.*
392 2015;162(2):328-337. doi:10.1016/j.cell.2015.06.012
- 393 6. Stokke C, Waldminghaus T, Skarstad K 2011. Replication patterns and organization of
394 replication forks in *Vibrio cholerae*. *Microbiology.* 157(3):695-708.
395 doi:10.1099/mic.0.045112-0
- 396 7. Quardokus EM, Brun YV. Cell cycle timing and developmental checkpoints in
397 *Caulobacter crescentus*. *Current Opinion in Microbiology.* 2003;6(6):541-549.
398 doi:10.1016/j.mib.2003.10.013
- 399 8. Nathwani D, Raman G, Sulham K, Gavaghan M, Menon V. Clinical and economic
400 consequences of hospital-acquired resistant and multidrug-resistant *Pseudomonas*
401 *aeruginosa* infections: a systematic review and meta-analysis. *Antimicrobial Resistance
402 and Infection Control.* 2014;3(1):32. doi:10.1186/2047-2994-3-32
- 403 9. Rossi E, La Rosa R, Bartell JA, et al. *Pseudomonas aeruginosa* adaptation and evolution
404 in patients with cystic fibrosis. *Nat Rev Microbiol.* 2021;19(5):331-342.
405 doi:10.1038/s41579-020-00477-5
- 406 10. La Rosa R, Johansen HK, Molin S. Convergent Metabolic Specialization through Distinct
407 Evolutionary Paths in *Pseudomonas aeruginosa*. *mBio.* 2018;9(2):e00269-18.
408 doi:10.1128/mBio.00269-18
- 409 11. Gibson B, Wilson DJ, Feil E, Eyre-Walker A. The distribution of bacterial doubling
410 times in the wild. *Proceedings of the Royal Society B: Biological Sciences.*
411 285(1880):20180789. doi:10.1098/rspb.2018.0789
- 412 12. Vallet-Gely I, Boccard F. Chromosomal Organization and Segregation in *Pseudomonas*
413 *aeruginosa*. *PLOS Genetics.* 2013;9(5):e1003492. doi:10.1371/journal.pgen.1003492
- 414 13. Bhowmik BK, Clevenger AL, Zhao H, Rybenkov VV. Segregation but Not Replication
415 of the *Pseudomonas aeruginosa* Chromosome Terminates at Dif. *mBio.*
416 2018;9(5):e01088-18. doi:10.1128/mBio.01088-18
- 417 14. Dötsch A, Schniederjans M, Khaledi A, et al. The *Pseudomonas aeruginosa*
418 Transcriptional Landscape Is Shaped by Environmental Heterogeneity and Genetic
419 Variation. *mBio.* 2015;6(4):e00749-15. doi:10.1128/mBio.00749-15
- 420 15. Tan J, Hammond JH, Hogan DA, Greene CS. ADAGE-Based Integration of Publicly
421 Available *Pseudomonas aeruginosa* Gene Expression Data with Denoising Autoencoders
422 Illuminates Microbe-Host Interactions. *mSystems.* 2016;1(1):e00025-15.
423 doi:10.1128/mSystems.00025-15
- 424 16. Hornischer K, Khaledi A, Pohl S, et al. BACTOME—a reference database to explore the
425 sequence- and gene expression-variation landscape of *Pseudomonas aeruginosa* clinical
426 isolates. *Nucleic Acids Research.* 2019;47(D1):D716-D720. doi:10.1093/nar/gky895

427 17. Selim H, Radwan TEE, Reyad AM. Regulation of T3SS synthesis, assembly and
428 secretion in *Pseudomonas aeruginosa*. *Arch Microbiol.* 2022;204(8):468.
429 doi:10.1007/s00203-022-03068-5

430 18. Mesaros N, Glupczynski Y, Avrain L, Caceres NE, Tulkens PM, Van Bambeke F. A
431 combined phenotypic and genotypic method for the detection of Mex efflux pumps in
432 *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy.* 2007;59(3):378-386.
433 doi:10.1093/jac/dkl504

434 19. Schuster M, Lostroh CP, Ogi T, Greenberg EP. Identification, Timing, and Signal
435 Specificity of *Pseudomonas aeruginosa* Quorum-Controlled Genes: a Transcriptome
436 Analysis. *Journal of Bacteriology.* 2003;185(7):2066-2079. doi:10.1128/JB.185.7.2066-
437 2079.2003

438 20. Chadha J, Harjai K, Chhibber S. Revisiting the virulence hallmarks of *Pseudomonas*
439 *aeruginosa*: a chronicle through the perspective of quorum sensing. *Environmental*
440 *Microbiology.* 2022;24(6):2630-2656. doi:10.1111/1462-2920.15784

441 21. Díaz-Pérez AL, Zavala-Hernández AN, Cervantes C, Campos-García J. The
442 gnyRDBHAL cluster is involved in acyclic isoprenoid degradation in *Pseudomonas*
443 *aeruginosa*. *Appl Environ Microbiol.* 2004;70(9):5102-5110.
444 doi:10.1128/AEM.70.9.5102-5110.2004

445 22. Herrick J, Sclavi B. Ribonucleotide reductase and the regulation of DNA replication: an
446 old story and an ancient heritage. *Molecular Microbiology.* 2007;63(1):22-34.
447 doi:10.1111/j.1365-2958.2006.05493.x

448 23. Margolin W. FtsZ and the division of prokaryotic cells and organelles. *Nat Rev Mol Cell*
449 *Biol.* 2005;6(11):862-871. doi:10.1038/nrm1745

450 24. Mathee K, Narasimhan G, Valdes C, et al. Dynamics of *Pseudomonas aeruginosa*
451 genome evolution. *Proceedings of the National Academy of Sciences.* 2008;105(8):3100-
452 3105. doi:10.1073/pnas.0711982105

453 25. Sobetzko P, Travers A, Muskhelishvili G. Gene order and chromosome dynamics
454 coordinate spatiotemporal gene expression during the bacterial growth cycle.
455 *Proceedings of the National Academy of Sciences.* 2012;109(2):E42-E50.
456 doi:10.1073/pnas.1108229109

457 26. Rossi E, Falcone M, Molin S, Johansen HK. High-resolution *in situ* transcriptomics of
458 *Pseudomonas aeruginosa* unveils genotype independent patho-phenotypes in cystic
459 fibrosis lungs. *Nat Commun.* 2018;9(1):3459. doi:10.1038/s41467-018-05944-5

460 27. Schulz S, Eckweiler D, Bielecka A, et al. Elucidation of Sigma Factor-Associated
461 Networks in *Pseudomonas aeruginosa* Reveals a Modular Architecture with Limited and
462 Function-Specific Crosstalk. *PLOS Pathogens.* 2015;11(3):e1004744.
463 doi:10.1371/journal.ppat.1004744

464 28. Lato DF, Golding GB. Spatial Patterns of Gene Expression in Bacterial Genomes. *J Mol*
465 *Evol.* 2020;88(6):510-520. doi:10.1007/s00239-020-09951-3

466 29. Tomasch J, Koppenhöfer S, Lang AS. Connection Between Chromosomal Location and
467 Function of CtrA Phosphorelay Genes in Alphaproteobacteria. *Frontiers in*
468 *Microbiology.* 2021;12. Accessed October 2, 2022.
469 <https://www.frontiersin.org/articles/10.3389/fmicb.2021.662907>

470 30. Rattray JB, Thomas SA, Wang Y, et al. Bacterial Quorum Sensing Allows Graded and
471 Bimodal Cellular Responses to Variations in Population Density. *mBio.*
472 2022;13(3):e00745-22. doi:10.1128/mbio.00745-22

473 31. Anetzberger C, Pirch T, Jung K. Heterogeneity in quorum sensing-regulated
474 bioluminescence of *Vibrio harveyi*. *Molecular Microbiology.* 2009;73(2):267-277.
475 doi:10.1111/j.1365-2958.2009.06768.x

476 32. Patzelt D, Wang H, Buchholz I, et al. You are what you talk: quorum sensing induces
477 individual morphologies and cell division modes in *Dinoroseobacter shibae*. *ISME J.*
478 2013;7(12):2274-2286. doi:10.1038/ismej.2013.107

479 33. Reck M, Tomasch J, Wagner-Döbler I. The Alternative Sigma Factor SigX Controls
480 Bacteriocin Synthesis and Competence, the Two Quorum Sensing Regulated Traits in
481 *Streptococcus mutans*. *PLOS Genetics*. 2015;11(7):e1005353.
482 doi:10.1371/journal.pgen.1005353

483 34. Kordes A, Preusse M, Willger SD, et al. Genetically diverse *Pseudomonas aeruginosa*
484 populations display similar transcriptomic profiles in a cystic fibrosis explanted lung. *Nat
485 Commun.* 2019;10(1):3397. doi:10.1038/s41467-019-11414-3

486 35. Jeske A, Arce-Rodriguez A, Thöming JG, Tomasch J, Häussler S. Evolution of biofilm-
487 adapted gene expression profiles in lasR-deficient clinical *Pseudomonas aeruginosa*
488 isolates. *npj Biofilms Microbiomes*. 2022;8(1):1-14. doi:10.1038/s41522-022-00268-1

489 36. Laub MT, McAdams HH, Feldblyum T, Fraser CM, Shapiro L. Global Analysis of the
490 Genetic Network Controlling a Bacterial Cell Cycle. *Science*. 2000;290(5499):2144-
491 2148. doi:10.1126/science.290.5499.2144

492 37. Waldbauer JR, Rodrigue S, Coleman ML, Chisholm SW. Transcriptome and Proteome
493 Dynamics of a Light-Dark Synchronized Bacterial Cell Cycle. *PLOS ONE*.
494 2012;7(8):e43432. doi:10.1371/journal.pone.0043432

495 38. De Nisco NJ, Abo RP, Wu CM, Penterman J, Walker GC. Global analysis of cell cycle
496 gene expression of the legume symbiont *Sinorhizobium meliloti*. *PNAS*.
497 2014;111(9):3217-3224. doi:10.1073/pnas.1400421111

498 39. Bandekar AC, Subedi S, Ioerger TR, Sassetti CM. Cell-Cycle-Associated Expression
499 Patterns Predict Gene Function in Mycobacteria. *Current Biology*. 2020;30(20):3961-
500 3971.e6. doi:10.1016/j.cub.2020.07.070

501 40. Pountain A, Jiang P, Podkowik M, Shopsin B, Torres VJ, Yanai I. A quantitative model
502 for the transcriptional landscape of the bacterial cell cycle. Published online October 23,
503 2022;2022.10.22.513359. doi:10.1101/2022.10.22.513359

504 41. Teeseling MCF van, Thanbichler M. Generating asymmetry in a changing environment:
505 cell cycle regulation in dimorphic alphaproteobacteria. *Biological Chemistry*.
506 2020;401(12):1349-1363. doi:10.1515/hzs-2020-0235

507 42. Liberati NT, Urbach JM, Miyata S, et al. An ordered, nonredundant library of
508 *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proceedings of the
509 National Academy of Sciences*. 2006;103(8):2833-2838. doi:10.1073/pnas.0511100103

510 43. Van P, Jiang W, Gottardo R, Finak G. ggCyto: next generation open-source visualization
511 software for cytometry. *Bioinformatics*. 2018;34(22):3951-3953.
512 doi:10.1093/bioinformatics/bty441

513 44. Bense S, Witte J, Preuße M, et al. *Pseudomonas aeruginosa* post-translational responses
514 to elevated c-di-GMP levels. *Molecular Microbiology*. 2022;117(5):1213-1226.
515 doi:10.1111/mmi.14902

516 45. Avraham R, Haseley N, Fan A, Bloom-Ackermann Z, Livny J, Hung DT. A highly
517 multiplexed and sensitive RNA-seq protocol for simultaneous analysis of host and
518 pathogen transcriptomes. *Nat Protoc.* 2016;11(8):1477-1491. doi:10.1038/nprot.2016.090

519 46. Freiherr von Boeselager R, Pfeifer E, Frunzke J. Cytometry meets next-generation
520 sequencing – RNA-Seq of sorted subpopulations reveals regional replication and iron-
521 triggered prophage induction in *Corynebacterium glutamicum*. *Sci Rep.* 2018;8(1):14856.
522 doi:10.1038/s41598-018-32997-9

523 47. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods*.
524 2012;9(4):357-359. doi:10.1038/nmeth.1923

525 48. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for
526 assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923-930.
527 doi:10.1093/bioinformatics/btt656

528 49. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential
529 expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-140.
530 doi:10.1093/bioinformatics/btp616

531 50. Futschik ME, Carlisle B. Noise-robust soft clustering of gene expression time-course
532 data. *J Bioinform Comput Biol*. 2005;03(04):965-988. doi:10.1142/S0219720005001375

533

534 **Figure Legends**

535

536 **Figure 1. Growth and Replication dynamics of *P. aeruginosa* in LB medium.** **(A)** Optical density
537 and cell numbers followed for 10 h of growth in LB medium. **(B)** Distribution of cell area as determined
538 from EM micrographs. **(C)** Distribution of chromosome content revealed by flow cytometric analysis of
539 SybrGreen fluorescence (One to four chromosome equivalents indicated by color). The lower panel
540 shows representative distributions of fluorescence intensity for up to 7 h. R indicates replicating cells
541 during coupled growth. **(D)** Representative EM micrographs of cells during uncoupled (3 h) and
542 coupled (10 h) growth. Visible division planes are marked by a white arrow.

543

544 **Figure 2. Transcriptome dynamics during growth in LB medium.** **(A)** Expression dynamics of the
545 eight clusters determined with mfuzz. Shown are the changes of the average expression in the
546 according clusters during the course of a 10h-cultivation. **(B)** Significantly ($p < 0.05$) enriched KEGG-
547 categories in the eight clusters. Size indicates the number of enriched genes in the category, color is
548 according to p-value.

549

550 **Figure 3. Global chromosomal gene expression changes between different growth phases.** **(A)**
551 Distribution of genes on the chromosomes that show the highest expression during uncoupled
552 (clusters 1-3), transition (clusters 4-6) and coupled (clusters 7-8) growth phases. **(B)** Expression of
553 genes located in the ori and ter proximal during uncouples (1-3 h), transition (4-5 h) and coupled (6-10
554 h) growth phases. **(C)** \log_2 FCs between time points from different growth phases. Red lines show the
555 fitted general additive models; orange lines show the models shifted up with the conserved region at
556 the terminus set to \log_2 FC of zero. Representative chromosome content indicative for the different
557 growth phases is shown on the right.

558

559 **Figure 4. Transcriptomes of replicating and non-replicating cells during coupled growth.** **(A)**
560 Sampling scheme for method evaluation. **(B)** Correlation between transcriptomes of differently treated
561 RNAs. Data for two additional replicates are shown in Supplementary Figure S4A. **(C)** Differential
562 expression between replicating (R) and non-replicating (C1, C2) cells. Number of significantly up- and
563 down-regulated genes between fractions (dark red) are shown in the left and right corner at the
564 bottom of each panel, respectively. **(D)** Chromosome-wide differential gene expression in replicating
565 (R) versus non-replicating (C1) cells. Genes that change significantly in expression are marked in
566 dark red. Operons discussed in the text are marked in yellow. The cell-division gene cluster is shown
567 above the plot. The red line shows a fitted general additive model. Data for the comparisons R vs. C2
568 and C2 vs. C1 is shown in Supplementary Figure S4B.

569

570

571 **Supplementary Figure Legends**

572

573 **Supplementary Figure S1. Flow cytometric determination of relative cell size and chromosome**
574 **content during growth in LB medium. (A)** Changes of the side scatter (SSC) indicates reductive
575 cell division from 3 h to 7 h cultivation time. **(B)** Changes in the distribution of chromosome content for
576 three biological replicates in the course of 10 h cultivation.

577

578 **Supplementary Figure S2. Transcriptome dynamics during growth in LB medium. (A)**

579 Multidimensional scaling (MDS) plot of samples taken during 10 h cultivation. Note the different timing
580 during the shift to coupled growth (6 h sample) for the two independent experiments. **(B)**
581 Determination of ideal number of clusters based on the minimum centroid distance within the clusters.
582 Increasing the number of clusters above 8 does not lead to further reduction of centroid distance. **(C)**
583 Expression profiles of genes in the 8 clusters determined with the mfuzz-package. The number of
584 genes within the cluster is shown below the cluster number. Cluster affiliation alongside expression
585 data is also documented in Supplementary Table S1.

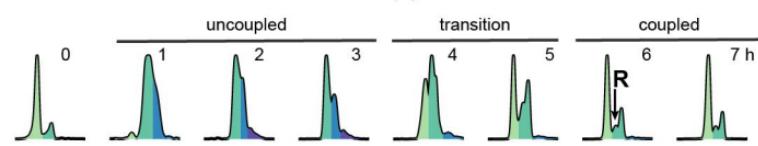
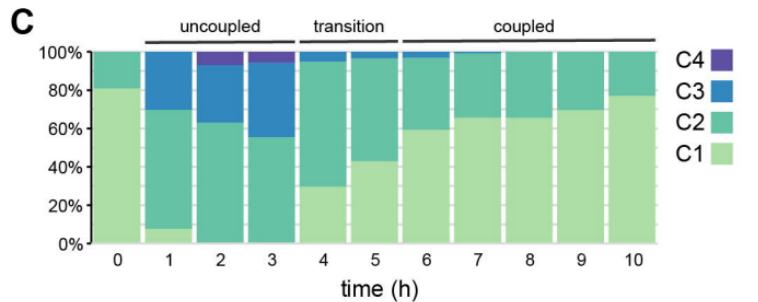
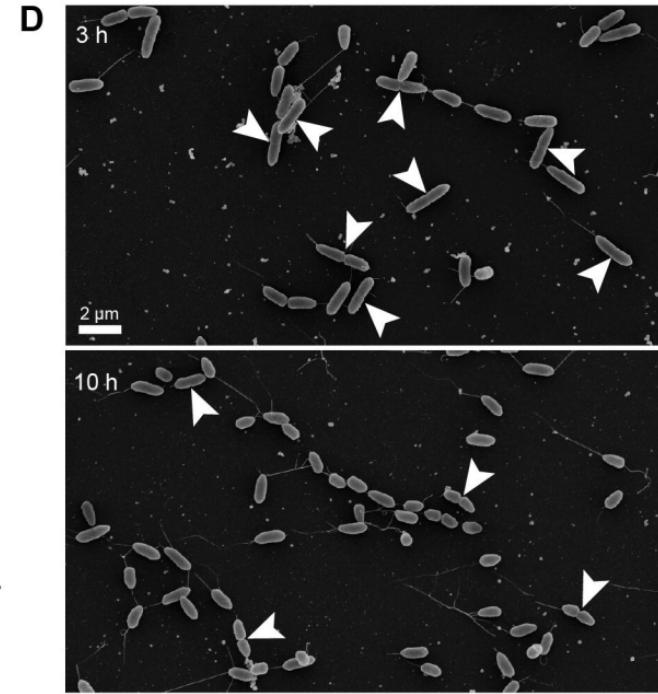
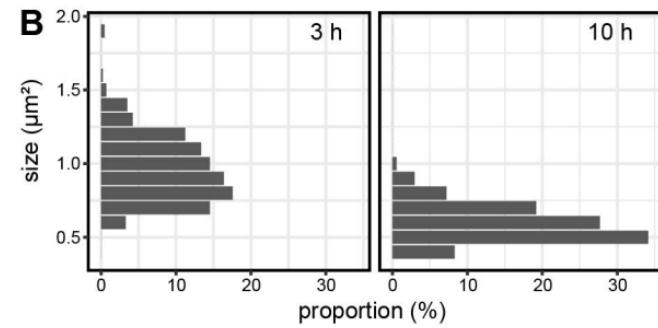
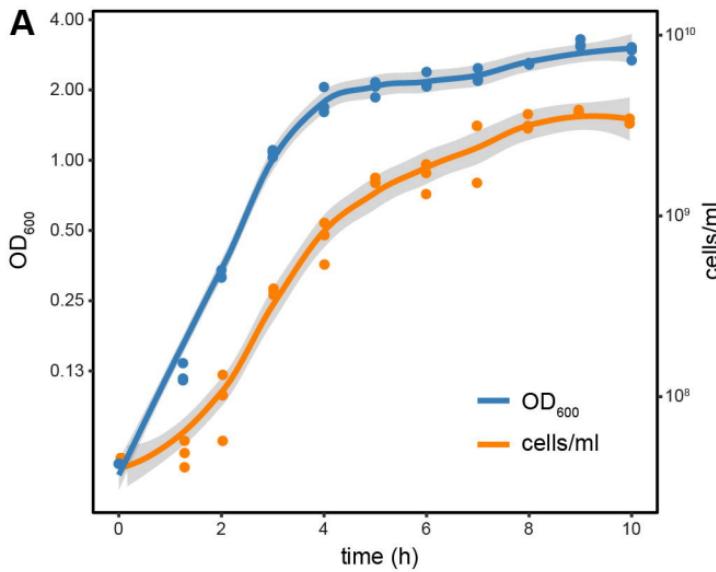
586

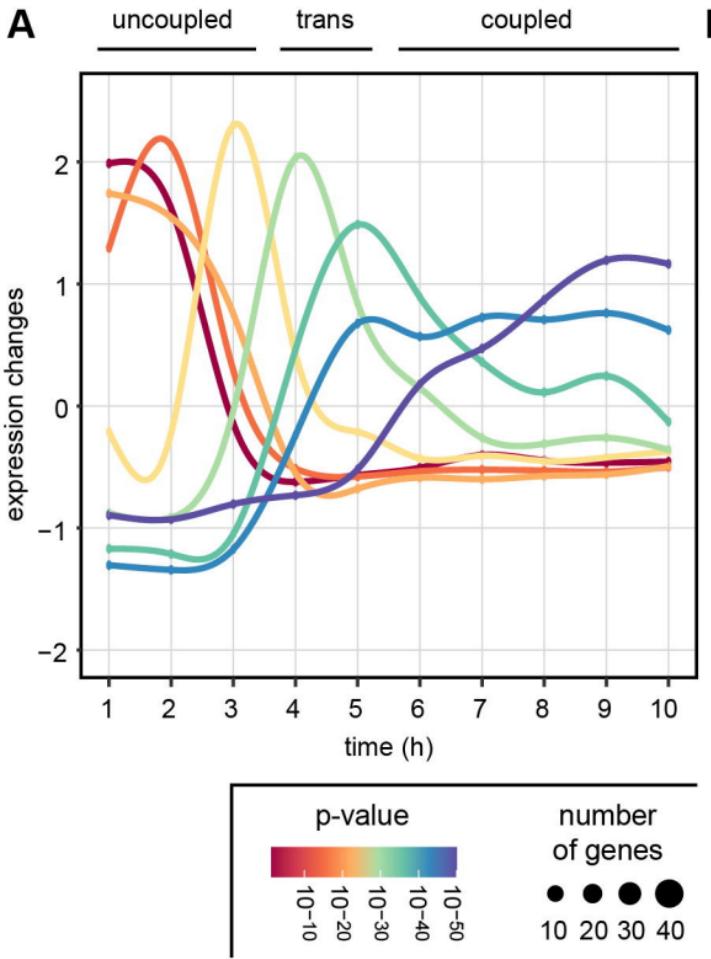
587 **Supplementary Figure S3. Time-resolved chromosomal gene expression changes during**
588 **growth in LB medium.** \log_2 fold changes between subsequent time points are shown. Red lines
589 show the fitted general additive models.

590

591 **Supplementary Figure S4. Transcriptomes of replicating and non-replicating cells during**
592 **coupled growth. (A)** Correlation between transcriptomes of differently treated RNAs (see Figure 4A).
593 **(B)** Chromosome-wide differential gene expression in replicating pre-divisinal (C2) versus non-
594 replicating (C1) and replicating (R) versus non-replicating cells. Genes that change significantly in
595 expression are marked in dark red. The red line shows a fitted general additive model.

596



A**B**