

# Trophic cooperation promotes bacterial survival of Staphylococcus aureus and Pseudomonas aeruginosa.

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#### 26 Abstract

27 In the context of infection, Pseudomonas aeruginosa and Staphylococcus aureus are frequently co-28 isolated, particularly in cystic fibrosis (CF) patients. Within lungs, the two pathogens exhibit a range of 29 competitive and coexisting interactions. In the present study, we explored the impact of S. aureus on 30 P. aeruginosa physiology in the context of coexistence. Transcriptomic analyses showed that S. aureus 31 affects significantly and specifically the expression of numerous genes involved in P. aeruginosa carbon 32 and amino acid metabolism. In particular, 65% of the strains presented an important overexpression of 33 the genes involved in the acetoin catabolic (aco) pathway. We demonstrated that acetoin is (i) produced 34 by clinical S. aureus strains, (ii) detected in sputa from CF patients and (iii) involved in P. aeruginosa's 35 aco system induction. Furthermore, acetoin is catabolized by P. aeruginosa, a metabolic way that 36 improves the survival of both pathogens by providing a new carbon source for P. aeruginosa and 37 avoiding toxic accumulation of acetoin on S. aureus. Due to its beneficial effects on both bacteria, 38 acetoin catabolism could testify to the establishment of trophic cooperation between S. aureus and 39 P. aeruginosa in the CF lung environment, promoting their persistence.

40 Introduction

41

42 Infectious sites constitute rich microbial ecosystems shared by a large diversity of 43 microorganisms, including the native microbiota and pathogens. Lungs of Cystic Fibrosis (CF) patients 44 are a well-known example of this microbial richness as they gather more than 60 genera of bacteria (1, 45 2). Such density of microorganisms promotes their interactions, themselves modeling their biological 46 activities and their environment (3, 4). However, microbial interactions are dynamic and range from 47 antagonism to cooperation according to species and environmental conditions (5). For instance, the 48 opportunistic pathogen Pseudomonas aeruginosa is well-known for its competitiveness in various 49 ecosystems thanks to many quorum-sensing-mediated factors such as phenazines, rhamnolipids or 50 type 6 secretion system (6). P. aeruginosa thus can alter growth, biofilm formation or respiration of 51 yeasts, fungi, Gram-negative and -positive bacteria (6). Among them, Staphylococcus aureus is 52 particularly sensitive to *P. aeruginosa* virulence factors and these can directly lyse staphylococci (7). 53 This competitive interaction between P. aeruginosa and S. aureus is observed for environmental but 54 also clinical strains as both pathogens are frequently co-isolated from wound and CF lung samples (7, 55 8). Until recently, the anti-staphylococcal behavior of P. aeruginosa was the only one observed between 56 the two species and was thus extensively described (7). In the context of CF lung infections, this 57 competitive interaction is highlighted by the decreased S. aureus prevalence as P. aeruginosa colonizes 58 lungs during adolescence (9). However, Baldan et al. first noted that a non-competitive state between 59 P. aeruginosa and S. aureus could establish during the development of CF chronic infections (10), 60 calling into question the antagonistic model between the two pathogens.

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62 The establishment of this particular non-competitive state between *P. aeruginosa* and *S. aureus* 63 seems to be linked to P. aeruginosa adaptation to the pulmonary ecosystem. In fact, selective pressures 64 present in CF lung environment, such as host immune system or antibiotic treatments, drive 65 P. aeruginosa isolates towards a low-virulent but high-resistant state (11–14). Major virulence factors 66 involved in quorum-sensing or motility are often mutated in *P. aeruginosa* chronic infection isolates, inducing a decrease of anti-staphylococcal factors production and then a non-competitive interaction 67 68 (15). In addition, a rewiring of metabolism networks and a decrease of its catabolic repertoire also 69 accompany P. aeruginosa adaptation to CF environment. This trophic specialization commonly leads to 70 a slowed growth of chronic isolates and thus a reduced competitive behavior towards shared resources

71 (16). Several independent studies thus observed this coexistence state between S. aureus and 72 P. aeruginosa isolated from chronic infections (10, 15, 17, 18). Briaud et al. recently demonstrated that 73 this interaction pattern appears to be more frequent than expected. Indeed, among the quarter of CF 74 patients co-infected by both pathogens, 65% were infected by a coexisting S. aureus-P. aeruginosa pair 75 (18, 19). Recent studies show that coexistence between P. aeruginosa and S. aureus could promote 76 their persistence throughout establishment of cooperative interaction. In these conditions, coexisting 77 bacteria demonstrated an increased tolerance to antibiotics: to tobramycin and tetracycline for S. aureus 78 and to gentamicin for P. aeruginosa; this appeared to be related to the induction of small colony variants 79 (15, 17, 18, 20). However, the effects of coexistence on bacterial general physiology, and not only 80 virulence-associated traits, have not been explored yet. Despite its significance in infectious ecosystem, 81 coexistence between P. aeruginosa and S. aureus remains thus poorly understood.

82

Using global and targeted transcriptomic approaches, we evaluated the impact of *S. aureus* presence on *P. aeruginosa* gene expression on a set of clinical pairs of strains isolated from CF coinfected patients. Coexistence with *S. aureus* induced the overexpression of many genes involved in utilization of alternative carbon sources in *P. aeruginosa*, such as amino acids and acetoin. Acetoin was shown to be produced by clinical *S. aureus* isolates *in vitro* and in CF sputum, and catabolized by *P. aeruginosa*. The beneficial effects of acetoin catabolism on both bacteria during their interaction highlight a trophic cooperation between *P. aeruginosa* and *S. aureus* in CF lung infections.

90

#### 91 Materials and Methods

92

#### 93 Bacterial strains

Bacterial strains and plasmids used in this study are listed in Table S1 and S2. CF clinical strains were isolated by the Infectious Agents Institute (IAI) from sputa of patients monitored in the two CF centers of Lyon hospitals (Hospices Civils de Lyon (HCL)). *S. aureus* and *P. aeruginosa* strains were isolated from patients co-infected by both bacteria. Each strain pair indicated in Table S1 was recovered from a single sample, obtained from different patients in most cases as indicated in Table S1. All the methods were carried out in accordance with relevant French guidelines and regulations. This study was submitted to the Ethics Committee of the HCL and registered under CNIL No 17-216. All patients
were informed of the study and consented to the use of their data.

As schematized in Figure S1A, interaction state was determined for each pair by growth inhibition tests on Tryptic Soy Agar (TSA) and in liquid cultures (18). As previously described, coexistence was characterized by: (i) an absence of inhibition halo on agar tests, and (ii) a similar growth in mono and co-culture during an 8 hours (18).

106 The knock-out  $\Delta acoR$  and  $\Delta aco$  mutants were generated in *P. aeruginosa* PA2600 strain via 107 allelic exchange thanks to suicide plasmids pEXG2 $\Delta acoR$  and pEXG2 $\Delta aco$  constructed as described 108 previously (21, 22). Gentamicin (Euromedex) was used at final concentrations of 50µg/ml. Detailed 109 protocols are given in supplementary data.

110

#### 111 Cultures conditions

112 Strains were grown in monoculture or co-culture in Brain-Heart Infusion (BHI) as described by 113 Briaud et al. (18). Briefly, S. aureus, P. aeruginosa, Burkholderia cenocepacia, Stenotrophomonas 114 maltophilia and Bacillus subtilis overnight precultures were diluted to OD<sub>600</sub> of 0.1 in BHI and grown 2h30 115 at 37°C and 200rpm. Cultures were then diluted to OD<sub>600</sub> of 2 in fresh medium and 10ml were mixed 116 with 10ml BHI for monocultures. Co-cultures were performed by mixing 10ml of P. aeruginosa 117 suspension with 10ml of S. aureus, B. cenocepacia, S. maltophilia or B. subtilis suspension. For 118 supernatant exposure, 10ml of S. aureus supernatant from a 4-hours culture were filtered on 0.22µM 119 filter and added to 10ml of P. aeruginosa suspension. Cultures were grown for 8 hours at 200rpm and 120 37°C for transcriptomic studies. Long-term survival assays were performed by extending incubation time 121 of S. aureus and P. aeruginosa mono- and co-cultures up to five days. Plating at day 0, 3 and 5 were 122 performed on mannitol salt agar (MSA, BBL<sup>™</sup> Difco) and cetrimide (Difco<sup>™</sup>) for S. aureus or 123 P. aeruginosa counts, respectively. For growth monitoring in presence of acetoin, minimal medium M63 124 (76mM (NH4)<sub>2</sub>SO<sub>4</sub>, 500mM KH<sub>2</sub>PO<sub>4</sub>, 9µM Fe.SO<sub>4</sub>.7H<sub>2</sub>O, 1mM MgSO<sub>4</sub>.7H<sub>2</sub>O) was inoculated with 125 P. aeruginosa to OD<sub>600</sub> of 0.1 and incubated for 25h at 37°C and 200rpm. Every two hours during 10 126 hours and at t=24h, acetoin was added to obtain a final concentration of 1.5mM in the culture. Plating 127 on TSA (Tryptic Soy Agar) was performed at t=0, one hour after each acetoin addition and at t=24h.

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#### 129 Genome sequencing and annotation

130 Genome sequencing and annotation of PA2596 and PA2600 strains were performed as 131 previously described (18). In order to compare CDS from PA2596 and PA2600, PAO1 strain 132 (NC\_002745.2) was used as a reference. Protein sequences were compared and grouped using a 133 similarity threshold of 95% through Roary (V3.8.2) (23). Gene names and numbers were gathered from 134 PAO1 and used as ID tag for common genes. For non-common genes, CDS from PA2596 and PA2600 135 were tagged with a specific name (gene number and name recovered from UniprotKB database). 136 Functional classification was performed using KEGG database and by manual literature check of each 137 gene function. The complete genome sequences for the PA2596 and PA2600 strains have been 138 deposited in GenBank under the accession number GCA 009650455.1 and GCA 009650545.1.

139

#### 140 Transcriptomic analysis

141 Figure S1 schematizes the global methodology used for transcriptomic analysis. RNAseq 142 analysis was performed on four pairs: the patient-specific pairs SA2597/PA2596 (competitive) and 143 SA2599/PA2600 (coexisting), and the crossed pairs SA2599/PA2596 (competitive) and 144 SA2597/PA2600 (coexisting). RNA extraction, cDNA libraries preparation and sequencing and reads 145 treatment were conducted as previously described (18). Gene expression was considered as 146 dysregulated when: (i) the fold change between co-culture and monoculture was at least 4-fold with an 147 adjusted P-value<0.05, (ii) the dysregulation was observed in the two pairs of strains with the same 148 interaction state, (iii) the dysregulation was specific to either coexistence or competition state. RNAseq 149 data that support our findings are available in the SRAdatabase under the BioprojectID: PRJNA562449, 150 PRJNA562453, PRJNA554085, PRJNA552786, PRJNA554237, PRJNA554233.

151

152 Confirmation of gene expression was achieved by RT-qPCR as previously described on 14 or 153 21 coexisting pairs including the SA2599/PA2600 couple used in RNAseq experiment **(Fig. S1)** (18). 154 Housekeeping genes *gyrB* and *rpoD* were used as endogenous control. Table S3 lists primers used and 155 target genes.

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#### 157 Acetoin and glucose dosages

158Acetoin was quantified using modified Voges-Proskauer test (24) optimized in 96-well159microplates. To 50µL of culture supernatant, 35µL of creatine (0.5% m/v, Sigma), 50µL of α-naphtol (5%

160 m/v, Sigma) and 50µL of KOH (40% m/v, Sigma) were sequentially added. The mix was incubated 15 161 minutes at room temperature and optical density at 560nm was read using spectrophometry (Tecan 162 Infinite Pro2000, Tecan-Switzerland). Glucose dosages were performed with glucose (Trinder, GOPOD) 163 assay kit (LIBIOS) in microplates. 185µL of dosage reagent were added to 5µL of culture supernatant 164 and incubated at 37°C for 5 minutes. Optical density at 540nm was measured. Acetoin and glucose 165 standard (Sigma) were performed in water, BHI or M63 according to experiment.

166

#### 167 Statistical analysis

168 Statistical analyses were performed using Prism GraphPad 8.0 software (San Diego, CA). 169 Differences in gene expression fold change and bacterial survival were studied using one-way ANOVA 170 with Dunnett's or Tukey's post-test comparisons, as specified in corresponding figures. Median acetoin 171 and glucose concentrations were compared through Mann-Whitney test or Kruskall-Wallis test with 172 Dunn's correction when appropriate. Differences were considered significant when *P*-values were lower 173 or equal to 0.05.

- 174
- 175 Results

176

#### 177 *P. aeruginosa* transcriptome is affected by *S. aureus* presence

178 We studied the genetic expression of P. aeruginosa in absence or presence of S. aureus in two 179 contexts: when P. aeruginosa and S. aureus were in competition or when they were in coexistence. We 180 thus performed RNAseq analyses using a competitive strain pair (SA2597/PA2596) and a coexisting 181 one (SA2599/PA2600). Each pair was recovered from a single sample of a co-infected CF patient 182 (Table S1). As nature of interaction is solely led by *P. aeruginosa* (18), the pairs were crossed to study 183 gene expression in additional competitive (SA2599/PA2596) and coexisting (SA2597/PA2600) pairs. 184 Transcriptomic effect was therefore evaluated during co-cultures of two competitive and two coexisting 185 strain pairs (Fig. S1). P. aeruginosa gene expression was considered dysregulated when dysregulation 186 was common to both co-cultures in comparison to monoculture. Each dysregulated gene was then 187 associated to a functional class thanks to a KEGG analysis (Fig. 1A, Tables S4 and S5).

189 Sixty-eight P. aeruginosa genes were dysregulated in co-culture in the context of competition, with 190 a majority of down-regulated genes (79.4%, Fig. 1A). Fifteen genes involved in nitrogen metabolism 191 and 19 genes involved in iron metabolism were down-regulated, making these two functional classes 192 the most affected in competition. Among these genes, the nir and nos systems involved in denitrification 193 but also genes implicated in iron uptake and transport (isc and fec genes) were down-regulated (Table 194 S4). Other functional classes were less affected, as only 4 genes linked to carbon and amino acids 195 metabolism (bauA, ddaR, gntK, arcD) and 3 genes encoding membrane and virulence factors (rfaD, 196 PA2412, cdrA) were dysregulated in presence of S. aureus.

197

198 More genes were affected during coexistence interaction, as a dysregulation of 105 genes was 199 observed in *P. aeruginosa* (Fig. 1A). In spite of a trend of up-regulation (56.4% of genes), we could 200 notice the down-regulation of 11 genes involved in nitrogen metabolism. Among these, 8 were also 201 down-regulated in the context of competition, especially genes from the nir system. We thus can 202 presume that the down-regulation of nir genes is not specific to coexistence or competition interaction 203 states. Conversely, the dysregulations of iron metabolism related genes appeared to be specific to 204 competition strains, as only one gene (*fhp*) of this functional class was down-regulated in coexistence. 205 However, coexistence specifically affected numerous genes belonging to functional classes of carbon 206 and amino acids metabolism (18 and 23 genes) and membrane and virulence factors (15 genes). 207 Concerning this last class, a trend towards lower expression was observed and probably due to the 208 down-regulation of membrane associated factors as the flp-tad system (flp, tad and rcpC genes 209 encoding Flp pilus) and the PA1874-1876 operon (encoding an efflux pump) (Table S5).

210

The most affected classes in coexistence with *S. aureus* were related to *P. aeruginosa* energetic metabolism (Fig. S2). We observed a down-regulation of genes coding for major pentose phosphate pathway enzymes, as the gluconokinase GntK, its regulator GntR and the operon *zwf-edaA* (*PA3183-PA3181*) encoding a glucose-6-phosphate 1-dehydrogenase, a 6-phosphogluconolactonase and a 2keto-3-deoxy-6-phosphogluconate aldolase (25). We also noticed the down-regulation of 5 other genes clustered near to this operon and involved in the same pathway (*edd* and *gapA* genes) or glucose transport (*gltB*, *gltF* and *gltK*) (25, 26).

219 In contrast, an up-regulation of numerous genes involved in the utilization of alternative carbon 220 sources as butanoate and amino acids was observed. The aco system, comprising the operon PA4148-221 PA4153 and the gene encoding its transcriptional regulator acoR (PA4147), was thus up-regulated in 222 P. aeruginosa coexisting with S. aureus. This system has been described in P. aeruginosa PAO1 to be 223 responsible for 2,3-butanediol and acetoin catabolism (27). According to KEGG analysis, the up-224 regulated genes acsA (PA0887), PA2555 (acs family) and hdhA (PA4022) are also involved in the 225 butanoate pathway and energy production from 2,3-butanediol and acetoin, as their products catalyse 226 the production of acetyl-coA from acetaldehyde and acetate (Fig. S2). Finally, 23 genes implicated in 227 amino acids metabolism were up-regulated in P. aeruginosa in presence of S. aureus. Most of them 228 were linked to the catabolism of several amino acids (Fig. S2). We can especially notice the *liu* operon 229 (PA2015-PA2012), the mmsAB operon (PA3569-PA3570) and the hut gene system (PA5097-PA5100), 230 involved in leucine, valine and histidine catabolism, respectively.

231

232 In order to confirm these transcriptomic effects, we co-cultivated a set of P. aeruginosa-233 S. aureus coexistence CF pairs and performed RT-gPCR to evaluate P. aeruginosa gene expression in 234 presence of S. aureus (Fig. 1B). Each pair was isolated from a single sputum. Twenty-six genes were 235 tested, including 19 identified as dysregulated during RNAseg analysis and belonging to the most 236 impacted categories, ie. carbon and amino acids metabolism, and membrane and virulence factors. 237 Most of the genes were tested in a total of 14 P. aeruginosa-S. aureus pairs; expression of four of these 238 genes was assessed in seven additional pairs to confirm the observed dysregulations. The different 239 P. aeruginosa strains have shown very different transcriptomic patterns during co-cultivation with 240 S. aureus, especially for membrane-associated and virulence factor genes. We noticed an over-241 expression of rcpC, tadA, tadG and flp from the flp-tad system from 52.4% to 35.7% of the P. aeruginosa 242 strains. We also tested 7 additional genes encoding virulence factors previously described as involved 243 in P. aeruginosa interaction with S. aureus as las, rhl, pch and pvd genes (7). Regarding these last 244 genes, no clear effect of S. aureus co-cultivation was observed.

245

However, clearer transcriptomic patterns were observed for genes linked to carbon and amino acids metabolism, the two most impacted gene classes during RNAseq experiment (**Fig. 1B**). We confirmed the up-regulation of *liuA* gene in 78.6% (11/14) of *P. aeruginosa* strains co-cultivated with S. aureus. We also confirmed the down-regulation of glucose metabolism genes in a high proportion of strains, ranging from 92.9% (13/14) for *zwf*, *gltF* and *edd* genes to 71.4% (10/14) for *gntK* gene. Finally, the up-regulation of genes involved in butanoate metabolism was confirmed for *acoR* (57.2%, 12/21), *PA4148* (66.7%, 14/21), *acoB* (57.1%, 8/14) and *PA4153* (64.3%, 9/14), suggesting an impact of coculture on the whole *aco* system in *P. aeruginosa*. In view of these results, we focused our study on four genes: *liuA* and *zwf*, respectively involved in leucine and glucose catabolism, *PA4148*, first gene of the *aco* operon and *acoR*, both responsible for acetoin catabolism.

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#### 7 *P. aeruginosa aco* system is induced by *S. aureus* acetoin

258 In order to determine if the transcriptomic dysregulations of acoR, PA4148, liuA and zwf in 259 P. aeruginosa PA2600 are specific to interaction with S. aureus, we tested the effect of three other 260 bacterial species: B. cenocepacia and S. maltophilia as they are sometimes associated with 261 P. aeruginosa in CF patients and Bacillus subtilis as it produces high amount of acetoin (28). Dosages 262 in B. cenocepacia and S. maltophilia monocultures confirmed that these bacteria do not produce acetoin 263 (Fig. S3) as previously described (29-31). We observed a down-regulation of P. aeruginosa zwf gene 264 expression in all co-cultures in comparison to monocultures (Fig. 2A). Regarding aco system genes 265 (acoR and PA4148) and liuA gene, only B. subtilis induced similar levels of overexpression than 266 S. aureus SA2599. We thus hypothesized that acetoin, produced by these two species during our 267 experiment (Fig. S3), may be the inductor signal for these genes during co-culture with S. aureus.

268

269 In order to test this hypothesis, we first explored whether an inductor signal was present in the 270 supernatant of S. aureus culture. We indeed observed an overexpression of acoR and PA4148 when 271 P. aeruginosa PA2600 was cultivated in S. aureus SA2599 culture supernatant, as well as the down-272 expression of zwf but to a lesser extent in comparison to co-culture (Fig. S4). On the contrary, we did 273 not observe overexpression of liuA gene, suggesting that this effect is not due to acetoin and requires 274 the presence of S. aureus cells. Secondly, we cultivated P. aeruginosa PA2600 in presence of S. aureus 275 UAMS-1 WT supernatant or its  $\Delta a$ /sSD derivative defective in acetoin synthesis (32). Supernatant of 276 wild-type UAMS-1 strain induced same transcriptomic patterns on P. aeruginosa as those obtained with 277 CF SA2599 strain (Fig. S4). In presence of UAMS-1 ΔalsSD supernatant, overexpression of aco genes 278 was almost totally abolished (Fig. 2B). However, acetoin addition to this supernatant restored this

overexpression in a dose-dependent manner but with threshold effects between 0.375mM and 1mM of acetoin (Fig. 2B). This indicates that induction through acetoin is one of the mechanisms involved in *aco* system overexpression in *P. aeruginosa*. According to this experiment, *zwf* gene down-regulation did not seem to be mediated by acetoin.

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#### 284 Coexisting isolates of *S. aureus* and *P. aeruginosa* efficiently metabolize acetoin

285

286 As the aco system is involved in acetoin catabolism in P. aeruginosa (27) and S. aureus 287 produces this molecule (27, 28), we hypothesized that *P. aeruginosa* could catabolize *S. aureus* acetoin. 288 To explore this hypothesis, we monitored acetoin concentration during mono- and co-cultures of three 289 pairs of strains: SA2599/PA2600 (Fig. 3A), SA146/PA146 and SA153/PA153A (Fig. S5A and B). 290 P. aeruginosa strains did not produce acetoin, but an acetoin accumulation up to 1.3mM was observed 291 in S. aureus monocultures. Interestingly, a reduction of acetoin accumulation of at least 30% was 292 observed when S. aureus was co-cultivated with P. aeruginosa, in comparison to S. aureus 293 monoculture. The same trend could be observed in CF patient sputa, as a higher acetoin concentration 294 was detected in sputa from S. aureus mono-infected patients (n=9) in comparison to sputa from 295 S. aureus-P. aeruginosa co-infected patients (n=11) (Fig. S6). This effect could be due to a down-296 regulation of S. aureus acetoin biosynthesis, or an acetoin catabolism in co-culture. Growth of 297 P. aeruginosa PA2600 in S. aureus SA2599 supernatant containing acetoin actually led to a reduction 298 of acetoin concentration, demonstrating the ability of *P. aeruginosa* to catabolize acetoin (Fig. 3B). This 299 ability was confirmed for the two other strains PA146 and PA153A when grown in supernatant of SA146 300 and SA153, respectively (Fig. S5C and D). We also noted that acetoin catabolism started after glucose 301 depletion in the supernatant and could be delayed by glucose addition (Fig. 3B). This suggests that 302 P. aeruginosa uses acetoin as an alternative carbon source in absence of easily available substrates 303 such as glucose. The early glucose depletion observed during co-culture supports this hypothesis 304 (Fig. 3A).

305

In order to test if this acetoin metabolism was specific to interaction state between *S. aureus* and *P. aeruginosa*, we evaluated acetoin production and catabolism for 12 couples of competition and
 12 couples of coexistence (Table S1). Cultivated in *P. aeruginosa* supernatant, *S. aureus* strains from

309 coexisting pairs were able to produce 4-times more acetoin (230µM) than strains from competitive pairs 310 (60µM) (Fig. 4A). Such distinction was not observed during culture in rich medium (Fig. S7). Regarding 311 P. aeruginosa, we cultivated the sets of competitive and coexisting P. aeruginosa strains in SA2599 312 supernatant and monitored acetoin catabolism (Fig. 4B). Both competitive and coexisting strains 313 catabolized acetoin since we observed a decrease in acetoin concentration for both groups. However, 314 coexisting strains showed an increased catabolism efficiency. Indeed, this group catabolized 98.6% of 315 acetoin after 4 hours of culture, while competitive strains catabolized only 47% of acetoin. This increased 316 efficiency of acetoin production and catabolism for coexisting strains could not be explained by a 317 difference in glucose utilization between competition and coexistence strains as both groups catabolized 318 glucose with the same efficiency (Fig. S8). Acetoin production by S. aureus and catabolism by 319 P. aeruginosa therefore seem to be more efficient in isolates from coexisting couples.

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#### 322 Acetoin catabolism by *P. aeruginosa* increases survival rates of both pathogens in co-culture

323 As P. aeruginosa catabolizes acetoin when medium is glucose-depleted, we tested the effect of 324 acetoin on PA2600 growth in minimal medium M63 (containing no glucose or amino acids) 325 supplemented with 1.5mM acetoin every 2 hours (Fig. 5). P. aeruginosa was able to grow up to 1.6×109 326 CFU/ml after 24h of culture with acetoin as sole carbon source while PA2600  $\Delta acoR$  and  $\Delta aco$  mutants 327 grew significantly less, reaching a maximum cell concentration of 1.5×10<sup>8</sup> UFC/ml at 24h (Fig. 5A). In 328 parallel, we quantified acetoin concentration. We observed an accumulation of acetoin all along the 329 experiment in presence of PA2600 \$\Delta acoR\$ and \$\Delta aco mutants. For WT strain, the accumulation of acetoin 330 reached its maximum at 5h of culture and afterwards slowly decreased to reach undetectable values at 331 10h of culture, demonstrating the consumption of acetoin in such conditions (Fig. 5B). A delay of 3h 332 between the start of acetoin catabolism and the growth augmentation of WT strain was noticed, and 333 might be due to metabolism adaptation. Acetoin catabolism nonetheless promoted a 10-fold increased 334 growth of *P. aeruginosa* during extended culture in glucose depleted medium.

335

336 In order to assess the impact of acetoin catabolism on survival of both pathogens, we co-337 cultivated *P. aeruginosa* PA2600,  $\Delta acoR$  and  $\Delta aco$  mutants with *S. aureus* SA2599. As *S. aureus* was 338 not able to grow in M63 poor medium and acetoin affects its long term survival (33, 34), we performed 339 a long term culture (5 days) in BHI medium. We determined the survival rate of S. aureus co-cultivated 340 with P. aeruginosa in comparison to monoculture (Fig. 6A). Co-culture with the WT strain of 341 P. aeruginosa induced a S. aureus survival rate of 4.7×10<sup>-1</sup> after 3 days of culture and of 5.1×10<sup>-3</sup> after 342 5 days. S. aureus survival thus appears to be highly affected by long-term co-culture with P. aeruginosa, 343 even if the strains stably coexist during shorter spans of culture (18). Interaction state with S. aureus 344 thus seems to rely on nutritional conditions, a fact already observed elsewhere (35, 36). However, the 345 survival rate of S. aureus was even lower during co-culture with P. aeruginosa  $\Delta acoR$  and  $\Delta aco$ 346 mutants, reaching only  $9.7 \times 10^{-2}$  at 3 days of culture and  $5.6 \times 10^{-4}$  at 5 days. S. aureus survival was thus 347 4 to 10 times lower when P. aeruginosa was not able to catabolize acetoin, in comparison to co-culture 348 with a strain that efficiently used the molecule. These results suggest that accumulation of acetoin may 349 impact S. aureus survival during co-culture with P. aeruginosa. In parallel, we determined the survival 350 rate of *P. aeruginosa* (Fig. 6B). We noticed that in such conditions, *P. aeruginosa* WT strain presented 351 a growth advantage in co-culture, with a maximum of 6.6-fold increase of *P. aeruginosa* population after 352 5 days in presence of S. aureus in comparison to monoculture (Fig. 6B). The opposite effect was 353 observed for aco mutant strains, as their populations were reduced by 75% for  $\Delta acoR$  and 32% for  $\Delta aco$ 354 after 3 days of co-culture in comparison to monoculture, confirming the role of acetoin catabolism in 355 favouring *P. aeruginosa* growth and survival.

356

357 In order to figure out if acetoin accumulation was involved in the decrease of S. aureus survival 358 rate, we monitored acetoin concentration in S. aureus monoculture and co-cultures (Fig. 6C). As 359 expected, we did not detect acetoin in co-culture with PA2600 WT strain over the five days but we 360 observed an accumulation of acetoin with PA2600  $\Delta acoR$  and  $\Delta aco$  mutants. The proportion of acetoin 361 in co-culture (1300µM to 1700µM/10<sup>6</sup> S. aureus) was more than 500 times higher than in monoculture 362 (2.5µM/10<sup>6</sup> S. aureus) (Fig. 6C). We thus cultivated S. aureus in different acetoin/cells proportions and 363 observed that acetoin had an inhibitory dose-dependent effect on S. aureus growth from 20µM/10<sup>6</sup> 364 S. aureus (Fig. S9). We concluded that acetoin accumulation may be responsible for the decreased 365 S. aureus survival during co-culture with PA2600 DacoR and Daco mutants. P. aeruginosa acetoin 366 catabolism thus allows a greater S. aureus survival during co-culture, in comparison to co-culture with 367 strains unable to catabolize acetoin.

Taken together, these results demonstrated that acetoin catabolism promotes *P. aeruginosa* survival as a nutritional alternative carbon source, and improves *S. aureus* survival during co-culture since a high concentration of acetoin appears to impair *S. aureus* growth.

373 Discussion

374

375 Coinfection with S. aureus and P. aeruginosa is a frequent situation especially in lungs of CF 376 patients, where coinfection accounts for 35% to 50% of cases (17, 18). In this context of coinfection, two 377 states of interaction between the two pathogens have been described: the well-known competitive 378 interaction where P. aeruginosa is able to inhibit the growth of S. aureus and the coexistence state 379 where growth of both species is not affected by each other. The first state has been extensively studied 380 and the leading bacterial determinants of S. aureus growth inhibition have been described (7). On the 381 contrary, little is known about the impact of coexistence state on bacterial physiology. In the present 382 study, we explored the impact of S. aureus on P. aeruginosa gene expression and physiology.

383 Comparing competitive and coexistence states, we observed that the down-regulation of genes 384 involved in iron metabolism was specific to competition. Most of these, such as fec genes or PA4467-385 PA4471 operon, are involved in ferrous iron uptake and down-regulated during iron-replete conditions 386 (37–39). These conditions are certainly generated by the lysis of S. aureus that provides an iron source 387 to P. aeruginosa during competitive interaction (7, 36), a situation not observed in coexistence. This 388 hypothesis is supported by the work of Tognon et al. (40) that also identified down-regulation of iron 389 metabolism genes during competitive interaction. Interestingly, they also noted typical responses of 390 amino acid starvation including the down-regulation of genes involved in branched-chain amino acid 391 degradation in competitive P. aeruginosa (40). While we did not identify such dysregulation in 392 competition, an overexpression of numerous genes involved in amino acid catabolism was noted during 393 coexistence (Table S5), emphasizing that these dysregulations depend on interaction state.

394

More interestingly, we observed that both carbon and amino acids metabolism was specifically affected during coexisting interaction. Many genes involved in glucose catabolism were down-regulated in coexisting isolates during co-culture with *S. aureus*, especially when the medium was glucosedepleted (**Fig. 3**). It is worth noting that the *zwf* gene, down-regulated in almost all *P. aeruginosa* tested strains, encodes a glucose-6-phosphate dehydrogenase that converts glucose- 6-phosphate to 6phosphogluconate; the first enzyme in the Entner-Doudoroff pathway, which is central to carbon metabolism in *Pseudomonas sp.* 

In such condition of glucose depletion, we demonstrated that *P. aeruginosa* was able to use an alternative carbon source provided by *S. aureus*: acetoin. Acetoin is a four-carbon molecule produced by the decarboxylation of  $\alpha$ -acetolactate. Owing to its neutral nature, production and excretion of acetoin during exponential growth prevents over acidification of the cytoplasm and the surrounding medium. When other carbon sources are exhausted, it can constitute an external energy source for fermentive bacteria (28).

408 Acetoin produced by S. aureus was shown to be an inductor of the aco operon and acoR 409 expression in P. aeruginosa (Fig. 2B) (27), allowing acetoin catabolism. This occurred in absence of 410 glucose and was potentially mediated by carbon catabolic repression (Fig. 3), a situation that was 411 already described in other bacteria such as B. subtilis (28). However, threshold effects in acetoin-412 mediated induction and variability in aco system overexpression in P. aeruginosa strains (Fig. 1B and 413 2B) suggest that other regulatory mechanisms may be involved. Our study may also support the 414 relationship between acetoin and branched-chain amino acid pathways. Indeed, the biosynthesis 415 pathways of acetoin and leucine are co-regulated and share the same precursor α-acetolactate in 416 S. aureus (28). In response to co-culture with S. aureus, P. aeruginosa clinical strains showed 417 overexpression of acetoin and leucine catabolism genes (Fig. 1B and 2), suggesting the presence of 418 both compounds in our co-culture conditions. All of our analyses were performed in vitro. However, 419 using Voges-Proskauer dosage, we were able to confirm the presence of acetoin in CF patient sputa, 420 and in lower concentrations for *P. aeruginosa*-positive samples (Fig. S6). No direct correlation between 421 P. aeruginosa presence and acetoin quantity can be established as other microorganisms present in 422 sputa may also have an impact on acetoin concentration. Nevertheless, our data support the work of 423 Španěl et al. (41) and suggest that P. aeruginosa may catabolize and use S. aureus acetoin in the lung 424 environment.

425

More importantly, we observed that catabolism of acetoin by *P. aeruginosa* and acetoin production by *S. aureus* were both more efficient for coexisting isolates, in comparison to competitive ones (Fig. 4). This underlines the adapted metabolic regulation in coexisting isolates in comparison to competitive ones. It is well known that the coexistence phenotype between *P. aeruginosa* and *S. aureus* is a consequence of an adaptation process. Indeed, *P. aeruginosa* strains isolated from early infection outcompete *S. aureus* while strains isolated from chronic infection are less antagonistic and can be co-

432 cultivated with *S. aureus* (7, 10, 17). It also has been widely described how both pathogens evolve
433 during colonization to evade the immune response and antibiotic treatment (42). Here, for the first time,
434 we suggest that evolution process leads to an adaptation of interspecies metabolic pathways between
435 *P. aeruginosa* and *S. aureus*.

436

437 Therefore, we suggest that acetoin produced by S. aureus could contribute to sputum nutritional 438 richness and be used by *P. aeruginosa* to survive in this nutritionally competitive environment during 439 chronic infection. This hypothesis is supported by the beneficial effect of acetoin catabolism on 440 P. aeruginosa growth and survival, especially during co-culture with S. aureus (Fig. 5 and 6B). 441 S. aureus survival in presence of P. aeruginosa was also shown to be highly affected by nutrient 442 availability induced by co-culture conditions (Fig. 6A). While coexistence is characterized by an absence 443 of S. aureus growth inhibition during 8-hour co-culture (18), it appears that nutritional competition can 444 still occur under unfavourable conditions and affect S. aureus survival. Therefore, coexistence between 445 the two pathogens is promoted in nutritionally rich environments, in line with previous observations (35, 446 36). Under adverse nutritional conditions induced by long-term culture, although its survival rate is 447 affected, acetoin catabolism benefits its producer, S. aureus (Fig. 6A). Although this effect seems to be 448 linked to acetoin accumulation in the medium as demonstrate by *P. aeruginosa*  $\Delta acoR$  and  $\Delta aco$ 449 mutants that do not catabolize acetoin anymore (Fig. 5B and 6C), the precise mechanism remains 450 unclear. In S. aureus, cell death in stationary phase may be induced by acetate production and ensuing 451 intracellular acidification. Thomas et al. showed that acetoin production counters cytoplasmic 452 acidification by consuming protons and promotes S. aureus survival in late-stationary phase (34). We 453 hypothesize that acetoin accumulation in the medium may induce a negative control of acetoin 454 synthesis, affecting *S. aureus* survival during co-culture conditions.

455

Previous studies demonstrated the potential benefits of *S. aureus* and *P. aeruginosa* during coinfection. For example, *S. aureus* facilitates the survival of *P. aeruginosa lasR* mutants commonly found in CF patients by detoxifying surrounding nitric oxide released by host immune cells (43). On the other hand, 4-hydroxy-2-heptylquinoline-N-oxide (HQNO) produced by *P. aeruginosa* cells inhibits respiration in *S. aureus* but also protects *S. aureus* cells from aminoglycosides (44). Additionally, we recently demonstrated that *S. aureus* antibiotic resistance and internalization into epithelial cells were

increased in presence of coexisting *P. aeruginosa* (18). Here, we show that carbon metabolism is largely
affected and that *P. aeruginosa* uses the acetoin produced by *S. aureus* as an alternative carbon source.
This metabolic dialogue between the two pathogens is selected during bacterial adaptation in CF lungs
and promotes their survival. Thereby, we highlight for the first time a trophic cooperation between *S. aureus* and *P. aeruginosa* during cooperative interaction.

467

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473

## 474 **Conflict of interest**

475 All authors declare no competing interests.

476

## 477 Ethical statement

All the strains used in this study were collected as part of the periodic monitoring of patients at the Hospices Civils de Lyon. This study was submitted to the Ethics Committee of the Hospices Civils de Lyon (HCL) and registered under CNIL No 17-216. All patients were informed of the study; however, as the study was non-interventional no written informed consent were required under local regulations.

484	References
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- 485 486
- Sibley CD, Rabin H, Surette MG. 2006. Cystic fibrosis: a polymicrobial infectious disease. Future
   Microbiol 1:53–61.
- 489 2. Guss AM, Roeselers G, Newton ILG, Young CR, Klepac-Ceraj V, Lory S, Cavanaugh CM. 2011.
- 490 Phylogenetic and metabolic diversity of bacteria associated with cystic fibrosis. ISME J 5:20–29.
- 491 3. Peters BM, Jabra-Rizk MA, O'May GA, Costerton JW, Shirtliff ME. 2012. Polymicrobial
- 492 interactions: impact on pathogenesis and human disease. Clin Microbiol Rev 25:193–213.
- 493 4. Murray JL, Connell JL, Stacy A, Turner KH, Whiteley M. 2014. Mechanisms of synergy in
  494 polymicrobial infections. J Microbiol Seoul Korea 52:188–199.
- 495 5. Abisado RG, Benomar S, Klaus JR, Dandekar AA, Chandler JR. 2018. Bacterial Quorum Sensing
  496 and Microbial Community Interactions. mBio 9.
- 497 6. Tashiro Y, Yawata Y, Toyofuku M, Uchiyama H, Nomura N. 2013. Interspecies interaction
  498 between Pseudomonas aeruginosa and other microorganisms. Microbes Environ 28:13–24.
- 499 7. Hotterbeekx A, Kumar-Singh S, Goossens H, Malhotra-Kumar S. 2017. In vivo and In vitro
  500 Interactions between Pseudomonas aeruginosa and Staphylococcus spp. Front Cell Infect
  501 Microbiol 7.
- Serra R, Grande R, Butrico L, Rossi A, Settimio UF, Caroleo B, Amato B, Gallelli L, de Franciscis
   S. 2015. Chronic wound infections: the role of Pseudomonas aeruginosa and Staphylococcus
   aureus. Expert Rev Anti Infect Ther 13:605–613.
- 505 9. O'Brien TJ, Welch M. 2019. Recapitulation of polymicrobial communities associated with cystic
  506 fibrosis airway infections: a perspective. Future Microbiol 14:1437–1450.
- 507 10. Baldan R, Cigana C, Testa F, Bianconi I, De Simone M, Pellin D, Di Serio C, Bragonzi A, Cirillo
- 508 DM. 2014. Adaptation of Pseudomonas aeruginosa in Cystic Fibrosis airways influences virulence
- 509 of Staphylococcus aureus in vitro and murine models of co-infection. PloS One 9:e89614.

510 11. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey
511 BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV. 2006. Genetic adaptation by
512 Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A
513 103:8487–8492.

514 12. Hoffman LR, Kulasekara HD, Emerson J, Houston LS, Burns JL, Ramsey BW, Miller SI. 2009.
515 Pseudomonas aeruginosa lasR mutants are associated with cystic fibrosis lung disease
516 progression. J Cyst Fibros Off J Eur Cyst Fibros Soc 8:66–70.

- 517 13. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, Molin S. 2012. Adaptation of
  518 Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective. Nat Rev
  519 Microbiol 10:841–851.
- Marvig RL, Sommer LM, Molin S, Johansen HK. 2015. Convergent evolution and adaptation of
   Pseudomonas aeruginosa within patients with cystic fibrosis. Nat Genet 47:57–64.
- 522 15. Limoli DH, Whitfield GB, Kitao T, Ivey ML, Davis MR, Grahl N, Hogan DA, Rahme LG, Howell PL,

523 O'Toole GA, Goldberg JB. 2017. Pseudomonas aeruginosa Alginate Overproduction Promotes

524 Coexistence with Staphylococcus aureus in a Model of Cystic Fibrosis Respiratory Infection. mBio
525 8.

- La Rosa R, Johansen HK, Molin S. 2019. Adapting to the Airways: Metabolic Requirements of
   Pseudomonas aeruginosa during the Infection of Cystic Fibrosis Patients. Metabolites 9.
- 528 17. Michelsen CF, Christensen A-MJ, Bojer MS, Høiby N, Ingmer H, Jelsbak L. 2014. Staphylococcus
  529 aureus alters growth activity, autolysis, and antibiotic tolerance in a human host-adapted
  530 Pseudomonas aeruginosa lineage. J Bacteriol 196:3903–3911.
- 531 18. Briaud P, Camus L, Bastien S, Doléans-Jordheim A, Vandenesch F, Moreau K. 2019.
- 532 Coexistence with Pseudomonas aeruginosa alters Staphylococcus aureus transcriptome,
- 533 antibiotic resistance and internalization into epithelial cells. Sci Rep 9.
- 534 19. Briaud P, Bastien S, Camus L, Boyadijian M, Reix P, Mainguy C, Vandenesch F, Doléans-
- 535 Jordheim A, Moreau K. 2020. Impact of coexistence phenotype between Staphylococcus aureus

- and Pseudomonas aeruginosa isolates on clinical outcomes among Cystic Fibrosis patients. Front
   Cell Infect Microbiol 10.
- 538 20. Frydenlund Michelsen C, Hossein Khademi SM, Krogh Johansen H, Ingmer H, Dorrestein PC,
- Jelsbak L. 2016. Evolution of metabolic divergence in Pseudomonas aeruginosa during long-term
   infection facilitates a proto-cooperative interspecies interaction. ISME J 10:1323–1336.
- 541 21. Carriel D, Simon Garcia P, Castelli F, Lamourette P, Fenaille F, Brochier-Armanet C, Elsen S,
- 542 Gutsche I. 2018. A Novel Subfamily of Bacterial AAT-Fold Basic Amino Acid Decarboxylases and
  543 Functional Characterization of Its First Representative: Pseudomonas aeruginosa LdcA. Genome
  544 Biol Evol 10:3058–3075.
- S45 22. Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ. 2005. ExsE, a secreted regulator of type III
  secretion genes in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 102:8006–8011.
- 547 23. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, Fookes M, Falush D, Keane
  548 JA, Parkhill J. 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinforma Oxf
  549 Engl 31:3691–3693.
- 550 24. Nicholson WL. 2008. The Bacillus subtilis ydjL (bdhA) Gene Encodes Acetoin Reductase/2,3551 Butanediol Dehydrogenase. Appl Environ Microbiol 74:6832–6838.
- 25. Lessie TG, Phibbs PV. 1984. Alternative pathways of carbohydrate utilization in pseudomonads.
  Annu Rev Microbiol 38:359–388.
- 26. Chevalier S, Bouffartigues E, Bodilis J, Maillot O, Lesouhaitier O, Feuilloley MGJ, Orange N,
- 555 Dufour A, Cornelis P. 2017. Structure, function and regulation of Pseudomonas aeruginosa
   556 porins. FEMS Microbiol Rev 41:698–722.
- 557 27. Liu Q, Liu Y, Kang Z, Xiao D, Gao C, Xu P, Ma C. 2018. 2,3-Butanediol catabolism in
- 558 *Pseudomonas aeruginosa* PAO1: 2,3-Butanediol catabolism in *Pseudomonas aeruginosa*.
  559 Environ Microbiol 20:3927–3940.
- 560 28. Xiao Z, Xu P. 2007. Acetoin Metabolism in Bacteria. Crit Rev Microbiol 33:127–140.

- 561 29. Gade N, Negi SS, Jindal A, Gaikwad U, Das P, Bhargava A. 2016. Dual Lower Respiratory Tract
  562 Infection by Burkholderia cepacia and Acinetobacter baumannii in A Neonate: A Case Report. J
  563 Clin Diagn Res JCDR 10:DD01–DD03.
- 30. Amoli RI, Nowroozi J, Sabokbar A, Rajabniya R. 2017. Isolation of Stenotrophomonas maltophilia
  from clinical samples: An investigation of patterns motility and production of melanin pigment.
  Asian Pac J Trop Biomed 7:826–830.
- 567 31. Dryahina K, Sovová K, Nemec A, Španěl P. 2016. Differentiation of pulmonary bacterial
- pathogens in cystic fibrosis by volatile metabolites emitted by their in vitro cultures: Pseudomonas
   aeruginosa, Staphylococcus aureus, Stenotrophomonas maltophilia and the Burkholderia cepacia
   complex. J Breath Res 10:037102.
- 32. Tsang LH, Cassat JE, Shaw LN, Beenken KE, Smeltzer MS. 2008. Factors Contributing to the
  Biofilm-Deficient Phenotype of Staphylococcus aureus sarA Mutants. PLoS ONE 3:e3361.
- 573 33. Chaudhari SS, Thomas VC, Sadykov MR, Bose JL, Ahn DJ, Zimmerman MC, Bayles KW. 2016.
- 574 The LysR-type transcriptional regulator, CidR, regulates stationary phase cell death in
- 575 Staphylococcus aureus: Metabolic control of cell death in S. aureus. Mol Microbiol 101:942–953.
- 576 34. Thomas VC, Sadykov MR, Chaudhari SS, Jones J, Endres JL, Widhelm TJ, Ahn J-S, Jawa RS,
- 577 Zimmerman MC, Bayles KW. 2014. A Central Role for Carbon-Overflow Pathways in the
- 578 Modulation of Bacterial Cell Death. PLoS Pathog 10:e1004205.
- 579 35. Miller CL, Van Laar TA, Chen T, Karna SLR, Chen P, You T, Leung KP. 2017. Global

580 transcriptome responses including small RNAs during mixed-species interactions with methicillin-

- resistant Staphylococcus aureus and Pseudomonas aeruginosa. MicrobiologyOpen 6.
- 582 36. Mashburn LM, Jett AM, Akins DR, Whiteley M. 2005. Staphylococcus aureus serves as an iron
- 583 source for Pseudomonas aeruginosa during in vivo coculture. J Bacteriol 187:554–566.
- 584 37. Visca P, Imperi F. 2018. An essential transcriptional regulator: the case of Pseudomonas

585 aeruginosa Fur. Future Microbiol 13:853–856.

- 38. Cornelis P, Matthijs S, Van Oeffelen L. 2009. Iron uptake regulation in Pseudomonas aeruginosa.
  Biometals Int J Role Met Ions Biol Biochem Med 22:15–22.
- 39. Ochsner UA, Wilderman PJ, Vasil AI, Vasil ML. 2002. GeneChip expression analysis of the iron
  starvation response in Pseudomonas aeruginosa: identification of novel pyoverdine biosynthesis
  genes. Mol Microbiol 45:1277–1287.
- 40. Tognon M, Köhler T, Luscher A, van Delden C. 2019. Transcriptional profiling of Pseudomonas
   aeruginosa and Staphylococcus aureus during in vitro co-culture. BMC Genomics 20:30.
- 593 41. Španěl P, Sovová K, Dryahina K, Doušová T, Dřevínek P, Smith D. 2016. Do linear logistic model
- analyses of volatile biomarkers in exhaled breath of cystic fibrosis patients reliably indicate

595 Pseudomonas aeruginosa infection? J Breath Res 10:036013.

- 42. Baishya J, Wakeman CA. 2019. Selective pressures during chronic infection drive microbial
  competition and cooperation. NPJ Biofilms Microbiomes 5:16.
- 43. Hoffman LR, Richardson AR, Houston LS, Kulasekara HD, Martens-Habbena W, Klausen M,
- Burns JL, Stahl DA, Hassett DJ, Fang FC, Miller SI. 2010. Nutrient availability as a mechanism for
  selection of antibiotic tolerant Pseudomonas aeruginosa within the CF airway. PLoS Pathog
  601 6:e1000712.
- 44. Hoffman LR, Déziel E, D'Argenio DA, Lépine F, Emerson J, McNamara S, Gibson RL, Ramsey
  BW, Miller SI. 2006. Selection for Staphylococcus aureus small-colony variants due to growth in
- the presence of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 103:19890–19895.
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#### 614 **Figure legends**

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616 Figure 1: Alteration of *P. aeruginosa* transcriptome induced by co-culture with *S. aureus*.

A. Number of under-expressed (grey bars) and over-expressed (black bars) genes of *P. aeruginosa* during co-culture with *S. aureus* for competitive (left) or coexisting (right) pairs. PA2596 competition and PA2600 coexistence strains were cultivated in absence or presence of SA2597 or SA2599 as described in Fig. S1. RNAs were extracted after 4 hours of culture and the RNAseq analysis was performed as described in material and methods. A gene was considered as differentially expressed when the Fold Change (FC) was >  $|2log_2|$  with an adjusted *P*-value <0.05. Functional classification was performed thanks to KEGG database and literature.

624 B. Fold change of 26 P. aeruginosa gene expression induced by co-culture with S. aureus during 625 coexistence interaction. Twenty-one S. aureus-P. aeruginosa coexisting pairs were isolated from 626 separate CF sputa recovered from 20 different patients. Each P. aeruginosa strain was cultivated in 627 absence or presence of its co-isolated S. aureus strain. RNAs were extracted after 4 hours of culture 628 and gene expression was assayed by RT-qPCR. A gene was considered as differentially expressed 629 when the Fold Change (FC) was > |2|, indicated by dotted lines. Black lines indicate the median. Genes 630 were tested in 14 (regular genes) or 21 (bold genes) strains. List of used strains is shown in Table S1. 631 Genes annotated with (\*) were not identified as dysregulated during the RNAseg experiment.

632

633 Figure 2: Fold changes of *P. aeruginosa acoR*, *PA4148*, *liuA* and *zwf* induced by culture 634 conditions.

A. Fold changes induced by co-culture with *S. aureus* (black bars), *B. subtilis* (grey bars), *B. cenocepacia* (hatched white bars) or *S. maltophilia* (hatched black bars). *P. aeruginosa* PA2600 strain was cultivated in absence or presence of *S. aureus* SA2599, *B. subtilis, B. cenocepacia* or *S. maltophilia*. RNAs were extracted after 4 hours of culture and gene expression was assayed by RTqPCR. Bars represent the mean fold change + SEM from three independent experiments. Dotted lines indicate a fold change = |2|. \**P*<sub>adj</sub><0.05, \*\**P*<sub>adj</sub><0.01, \*\*\**P*<sub>adj</sub><0.001 ANOVA with Dunnett's correction (*S. aureus vs.* condition). 642 B. Fold changes induced by culture in supernatant of S. aureus UAMS1 wild type (WT, black 643 bars),  $\Delta a$ /sSD mutant (white bars),  $\Delta a$ /sSD mutant complemented with increasing acetoin 644 concentrations (grey bars). P. aeruginosa PA2600 strain was cultivated in absence or presence of 645 filtered supernatant from S. aureus UAMS-1 WT, AalsSD or AalsSD complemented with acetoin 646 concentrations ranging from 0.375mM to 2mM. RNAs were extracted after 4 hours of culture and gene 647 expression was assayed by RT-qPCR. Bars represent the mean fold change + SEM from three 648 independent experiments. Dotted line indicates a fold change = |2|. \* $P_{adj}$ <0.05, \*\* $P_{adj}$ <0.01 ANOVA with 649 Tukey's correction.

650

Figure 3: Monitoring of acetoin (black lines) and glucose (grey lines) concentrations in *S. aureus* and *P. aeruginosa* monocultures or co-culture (A) or in *S. aureus* supernatant inoculated with
 *P. aeruginosa* (B), during coexisting interaction.

**A.** *S. aureus* SA2599 and *P. aeruginosa* PA2600 were cultivated in monoculture or co-culture. Acetoin and glucose were quantified from supernatant each hour. Points represent the mean acetoin or glucose concentration  $\pm$  SEM from three independent experiments. Similar results of acetoin accumulation are shown in Figure S5 for couples 146 and 153A.

**B.** A 4-hours filtered supernatant of *S. aureus* SA2599 was inoculated with *P. aeruginosa* PA2600 culture or sterile medium for controls. Supernatant was used unaltered (dotted lines) or complemented with glucose (solid lines). Acetoin and glucose were quantified from supernatant each hour. Points represent the mean acetoin or glucose concentration  $\pm$  SEM from three independent experiments. Similar results of acetoin catabolism are shown in Figure S5 for couples 146 and 153A.

663

Figure 4: Ability of acetoin production by *S. aureus* (A) and catabolism by *P. aeruginosa* (B) for
 competitive (grey bars) or coexisting (black bars) strains.

A. Acetoin concentration in *P. aeruginosa* supernatant inoculated with *S. aureus* strains from competition and coexistence couples. Each *S. aureus* strain from competition (n=12) and coexistence (n=12) couples was cultivated in *P. aeruginosa* PA2600 filtered supernatant and acetoin was quantified from supernatant after 6 hours of culture. Bars represent the median acetoin concentration  $\pm$  95% Cl. \*\*\*\**P*<0.0001 Mann-Whitney test. B. Acetoin concentration in *S. aureus* supernatant inoculated with *P. aeruginosa* strains from competition and coexistence couples. Each *P. aeruginosa* strain from competition (n=12) and coexistence (n=12) couples was cultivated in *S. aureus* SA2599 filtered supernatant and acetoin was quantified from supernatant after 4 hours of culture. Sterile supernatant was used as control for acetoin degradation. Bars represent the median acetoin concentration  $\pm$  95% CI. Dotted line indicate the initial acetoin concentration. \**P*<sub>adj</sub><0.05, \*\**P*<sub>adj</sub><0.01 Kruskall-Wallis test with Dunn's correction.

677

Figure 5: Monitoring of *P. aeruginosa* growth (A) and acetoin concentration (B) in minimal
medium supplemented in acetoin. *P. aeruginosa* PA2600 WT, Δ*acoR* and Δ*aco* strains were grown
in M63 medium and 1.5mM acetoin was added every 2 hours, indicated by black arrows.

A. Cultures were plated on TSA each 2 hours to count bacteria. Points represent the mean bacterial
 concentration *±* SEM from three independent experiments.

683 B. Acetoin was quantified from supernatant each hour. Points represent the mean acetoin concentration
 684 ± SEM from three independent experiments.

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Figure 6: Monitoring of *S. aureus* survival (A), *P. aeruginosa* survival (B) and acetoin concentration (C) during long-term co-culture. *S. aureus* SA2599 was cultivated in presence of *P. aeruginosa* PA2600 WT,  $\Delta acoR$  and  $\Delta aco$  during 5 days. Cultures were plated at J0, J3 and J5 on MSA and cetrimide to count *S. aureus* and *P. aeruginosa* respectively and acetoin was quantified from supernatant.

692 **A, B.** Survival rate was estimated by dividing the bacterial concentration in co-culture by bacterial 693 concentration in monoculture for each bacterium. Bars represent the mean survival rate + SEM from 694 five independent experiments.  $*P_{adj}<0.05$ ,  $****P_{adj}<0.0001$  one-way ANOVA with Dunnett correction (WT 695 vs. condition).

696 **C.** Acetoin concentration was normalized to *S. aureus* counts. Bars represent the median acetoin 697 concentration per 10<sup>6</sup> *S. aureus*  $\pm$  95% CI from five independent experiments. \**P*<sub>adj</sub><0.05, \*\**P*<sub>adj</sub><0.01, 698 Kruskal-Wallis with Dunn's correction.

699

700



Down-regulation
 Up-regulation







Acetoin concentration (mM)



- S. aureus monoculture
- P. aeruginosa monoculture
- Co-culture \*



S. aureus supernatant + glucose

6

4

2 Muu) (

concentration





✤ P. aeruginosa WT P. aeruginosa ∆acoR



# Trophic cooperation promotes bacterial survival of Staphylococcus aureus and

### Pseudomonas aeruginosa.

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#### Supplementary data

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**Figure S4:** Fold change of *P. aeruginosa acoR*, *PA4148*, *liuA* and *zwf* gene expression induced by culture with *S. aureus* or its supernatant.

**Figure S5:** Monitoring of acetoin concentration in *S. aureus* and *P. aeruginosa* monocultures or co-culture or in *S. aureus* supernatant inoculated with *P. aeruginosa*, for the pairs SA146/PA146 and SA153/PA153A.

Figure S6: Acetoin concentration in CF sputa from patients.

**Figure S7:** Acetoin concentration in cultures of *S. aureus* strains from competition and coexistence couples. **Figure S8:** Glucose concentrations in cultures of *S. aureus* and *P. aeruginosa* strains from competition and coexistence pairs.

Figure S9: Growth kinetic of S. aureus cultivated in absence or presence of acetoin.

#### Materials and methods

**Generation of PA2600 knock-out**  $\Delta acoR$  and  $\Delta aco$  mutants: upstream and downstream flanking regions of *acoR* and *aco* operon (474 bp and 486 bp fragments for *acoR*; 654 bp and 708 bp for *aco*) were PCR amplified (GoTaq polymerase, Promega) and cloned into pEXG2 by Sequence Ligation and Independent Cloning (SLIC) method (1,2). Resulting plasmids pEXG2-*acoR* and pEXG2-*aco* were then transferred into PA2600 by triparental mating. A first conjugation was performed between the two *E. coli* strains carrying either the pRK2013 helper plasmid or the constructed pEXG2 plasmid by spotting 30µL of pre-culture of each strain on LB plates. After two hours at 37°C, 30µL of PA2600 pre-culture were added on the dried spot and the plate was incubated five hours at 37°C. The spot was then re-suspended in LB medium and plated on Cetrimide plates supplemented with gentamycin. Resulting clones were then plated on LB containing 10% sucrose to select for plasmid excision by crossing-over. The resulting strains were checked for gentamicin sensitivity and gene deletion by PCR.

CF clinical strains	CF patient	Strain name	Interaction state	Experiments	Reference
<i>P. aeruginosa</i> (PA) and <i>S. aureus</i> (SA)	1	PA2596	Competition (SA2599, SA2597)	RNAseq	3
		SA2597		RNAseq	3
	2 (∎)	PA2600	Coexistence (SA2599, SA2597)	RNAseq; qRT-PCR screening; <i>aco</i> induction qRT- PCR; Acetoin monitoring in co-culture; Acetoin catabolism in SA supernatant; Culture in minimal medium; 5-day co-cultures	3
		SA2599		RNA seq; qRT-PCR screening; <i>aco</i> induction qRT- PCR; Acetoin production screening; Acetoin monitoring in co-culture; Production of SA supernatant; 5 days co-cultures; Growth in presence of acetoin	3
	3	PA7A SA7	Coexistence	qRT-PCR screening qRT-PCR screening	This study This study
	4	PA13 SA13	Coexistence	qRT-PCR screening qRT-PCR screening	This study This study
	5 (•)	PA27 SA27	Coexistence	qRT-PCR screening qRT-PCR screening	3 3
	6	PA30 SA30	Coexistence	qRT-PCR screening qRT-PCR screening	3 3
	7	PA31 SA31	Coexistence	qRT-PCR screening qRT-PCR screening	3 3
	8	PA37 SA37	Coexistence	qRT-PCR screening qRT-PCR screening	This study This study
	9	PA42 SA42	Coexistence	qRT-PCR screening qRT-PCR screening	3 3
	10	PA48 SA48	Coexistence	qRT-PCR screening qRT-PCR screening	This study This study
	11	PA53 SA53	Coexistence	qRT-PCR screening qRT-PCR screening	This study This study
	12	PA54 SA54	Coexistence	qRT-PCR screening qRT-PCR screening	This study This study
	13	PA69 SA69	Coexistence	qRT-PCR screening qRT-PCR screening	3 3
	14 (▲)	PA80 SA80	Coexistence	qRT-PCR screening qRT-PCR screening	3 3
	15	PA82 SA82	Coexistence	qRT-PCR screening qRT-PCR screening	3 3
	16	PA146	Coexistence	qRT-PCR screening; Acetoin catabolism screening; Acetoin monitoring in co-culture; Acetoin catabolism in SA supernatant	3
		SA146		qRT-PCR screening; Acetoin production screening; Acetoin monitoring in co-culture; Production of SA supernatant	3
	14 (▲)	PA148B SA148	Coexistence	qRT-PCR screening; Acetoin catabolism screening qRT-PCR screening; Acetoin production screening	3 3
	17	PA152 SA152	Coexistence	qRT-PCR screening; Acetoin catabolism screening qRT-PCR screening; Acetoin production screening	3 3
	18	PA153A	Coexistence	qRT-PCR screening; Acetoin catabolism screening; Acetoin monitoring in co-culture; Acetoin catabolism in SA supernatant	3
		PA153B SA153		Transcriptomic (qRT-PCR) qRT-PCR screening; Acetoin production screening; Acetoin monitoring in co-culture; Production of SA supernatant	3 3

	19	PA154A PA154B SA154	Coexistence	qRT-PCR screening; Acetoin catabolism screening qRT-PCR screening; Acetoin catabolism screening qRT-PCR screening; Acetoin production screening	3 3 3
	20	PA156 SA156	Coexistence	qRT-PCR screening; Acetoin catabolism screening qRT-PCR screening; Acetoin production screening	3 3
	21	PA166A SA166	Coexistence	qRT-PCR screening; Acetoin catabolism screening qRT-PCR screening; Acetoin production screening	3 3
	22	PA167 SA167	Competition	Acetoin catabolism screening Acetoin production screening	3 3
	2 (■)	PA171A SA171	Coexistence	Acetoin catabolism screening Acetoin production screening	3 3
	23	PA172 SA172	Competition	Acetoin catabolism screening Acetoin production screening	This study This study
	24	PA178 SA178	Coexistence	Acetoin catabolism screening Acetoin production screening	3 3
	25	PA179 SA179	Competition	Acetoin catabolism screening Acetoin production screening	3 3
	26	PA181 SA181	Competition	Acetoin catabolism screening Acetoin production screening	3 3
	27 (•)	PA186 SA186	Competition	Acetoin catabolism screening Acetoin production screening	3 3
	28	PA187	Competition	Acetoin catabolism screening	This study
	29	PA188A SA188	Competition	Acetoin catabolism screening Acetoin production screening	This study This study
	30	PA193A SA193	Competition	Acetoin catabolism screening Acetoin production screening	This study This study
	5 (•)	PA194A PA194B SA194	Coexistence	Acetoin catabolism screening Acetoin catabolism screening Acetoin production screening	This study This study This study
	31	PA197 SA197	Competition	Acetoin catabolism screening Acetoin production screening	This study This study
	32 33	SA198 PA199A PA199C	Competition Competition	Acetoin production screening Acetoin catabolism screening Acetoin catabolism screening	This study This study This study
	34	PA200 SA200	Competition	Acetoin catabolism screening Acetoin production screening	This study This study
	35	SA205	Competition	Acetoin production screening	This study
	36	SA207	Coexistence	Acetoin production screening	This study
	27 (♦)	SA213	Competition	Acetoin production screening	This study
B. cenocepacia	37	LUG2886	Coexistence (PA2600)	qRT-PCR (aco induction)	This study
S. maltophilia	38	LUG2884	Coexistence (PA2600)	qRT-PCR ( <i>aco</i> induction)	This study

Table S1: Clinical CF strains used in this study. Unless indicated, interaction state was tested between strains from the same clinical pair (ie. isolated from a single patient). Four strain pairs were recovered from a same patient but at different time points and are annotated with identical patient number and symbol (∎, ●, ▲ or ♦).

Strains /	News	Ohernesteristics	Interaction	<b>F</b>	Deferreres
plasmids	Name	Characteristics	state	Experiments	Reference
P. aeruginosa	PA2600 Δ <i>acoR</i>	acoR deletion mutant	Coexistence (SA2599)	Culture in minimal medium; 5- day co-cultures	This study
	PA2600 Δ <i>aco</i>	aco operon deletion mutant	Coexistence (SA2599)	Culture in minimal medium; 5- day co-cultures	This study
S. aureus	SA UAMS-1	WT strain	Coexistence (PA2600)	Production of SA supernatant ( <i>aco</i> induction)	4
	SA UAMS-1 ΔalsSD	UAMS-1489, <i>alsSD</i> deletion mutant	Coexistence (PA2600)	Production of SA supernatant (aco induction)	4
B. subtilis	LUG2953	WT strain	Coexistence (PA2600)	qRT-PCR ( <i>aco</i> induction)	This study
Plasmids	pEXG2	Gm <sup>R</sup> ; mobilizable, non- replicative vector in <i>P.</i> <i>aeruginosa</i>		acoR and aco deletions	2
	pEXG2-∆acoR	pEXG2 carrying upstream and downstream sequences of <i>acoR</i> for gene deletion		acoR deletion	This study
	pEXG2-∆aco	pEXG2 carrying upstream and downstream sequences of <i>aco</i> for operon deletion		aco deletion	This study
	pRK2013	Km <sup>R</sup> , helper plasmid with conjugative properties		acoR and aco deletions	5

**Table S2: Non-CF strains and plasmids used in this study.** *P. aeruginosa*  $\Delta acoR$  and  $\Delta aco$  mutants were constructed from the clinical CF isolate PA2600 (Table S1). Interaction state was tested with the strain indicated in brackets.

Use	Name	Sequence	Target	Amplicon Sizo
qPCR	PArpoD-F	GCGCAACAGCAATCTCGTCT	rpoD	177
	PArpoD-R	ATCCGGGGCTGTCTCGAATA		
	PAgyrB-F	ATCTCGGTGAAGGTACCGGA	gyrB	160
	PAgyrB-R	TGCCTTCGTTGGGATTCTCC	0,	
	OLC8-F	GCGAGGATCTCTACTTCCGC	acoR	140
	OLC8-R	CTCACCGAGTTCGATGCGTA		
	OLC9-F	GCGGATCGTCAACCTGTCAT	PA4148	86
	OLC9-R	CGATCACGGCAAACTTCGAG		
	OLC14-F	GTTCTTCAGGCTCCAGTCGG	pvdS	81
	OLC14-R	TTGCGGACGATCTGGAACAG	<i>μ</i> · · · · ·	
	OLC16-F	CGCGACAAGAGCGAATACCT	lasA	71
	OLC16-R	AGGGTCAGCAACACTTTCGG		
	OLC17-F	CTGGACTGAACCAGGCGATG	rhlA	113
	OLC17-R	CAGGTATTTGCCGACGGTCT		
	OLC22-F	CGACGGTATCCAGGTCGATG	PA1874	70
	OL C22-R	GAACTTGACCGTGACCACCT		10
	OL C23-F	CGGTGTTGCTCGGATACCTC	rcpC	120
	OL C23-R	GCTTGCGTTCGAGCTTTTCC		120
	OL C25-F	ATCGCCTGCTTCCAGTTGTC	pchD	166
	OL C25-R	AGAGAGTGAAGTTGTGCGCC	pone	100
	OL C29-F	GAGCGACGAACTGACCTACC	rhlB	200
	OL C29-R	TACTTCTCGTGAGCGATGCG		200
	OL C31-F	TGCTCACTTCGCTATGGACC	acoB	117
	OL C31-R	AGAAGATCACCGGGTCGTTG	4002	
	OL C33-F		PA4153	83
	OL C33-R	GACGATTICTICCAGGCCGA	171100	00
	OL C35-F	GCCCATCGAGTTCCGTATGT	bauD	151
	OL C35-R	GAGGCAGACGGTGAAGATCG		
	OL C39-F	GACGAAGACGGCATGAACCT	tadA	119
	OL C39-R	TCCAGTTCGTAGCGGGGAGAT		110
	OI C41-F	CCACCACGAAACTGCAACTG	tadG	107
	OL C41-R	GCTTCTCCAACCGAAGTCCA		
	OL C54-F	TTGCCGTATTGAGTCCCACG	fln	77
	OL C54-R	GACTITITCGCCGACTCCGT	nρ	
	OLC56-F	GGACTGACGCTCAGGCAAT	rhlC	74
	OL C56-R			
	OLC71-F	CAGCCGGACGAAGGTATCTC	zwf	113
	OLC71-R	GCGTGGTAGGTCTCGGAAAA		
	OLC72-F	GCTATACCCGCTCAAGGGC	dauA	92
	OL C72-R	TCTTGCGGTCGTAATCGGTG	agan	02
	OL C73-F	CTTCGCCTACCTGTTCAGCC	PA5099	163
	OLC73-R	CGGCAGGTAGCGTGAATAGT		
	OLC74-F	GCAGTTCGGAGCACATCAAC	PA1874	130
	OLC74-R	TCGATGCTGACGAAATCGGT		
	OL C75-F	TGGTATCCGGCGAACACATC	liuA	127
	OLC75-R	CACATCTTGCTGCCGTTGAG		· <b>_ ·</b>
	OLC92-F	CAGCCAGGACTACGAGAACG	lasR	153

	OLC92-R	TGGTAGATGGACGGTTCCCA		
	OLC93-F	GGCATTCCCCTCACCGAC	gntK	181
	OLC93-R	GGGTCAGTTCCAGGTAGACGA		
	OLC94-F	TGGAAGTGGCTGCTCAATCC	gltF	81
	OLC94-R	CCAGTCGAAGCGGAAACCTT		
	OLC95-F	CACCTGCACCTTCTATGGCA	edd	96
	OLC95-R	GTGTTCGGGTTGACGAAGGA		
	OLC96-F	CAACGGCACCTCGATCTTCA	mmsA	94
	OLC96-R	AATCGGGATGTTGATGCCCA		
Cloning	OLC58-F	GGTCGACTCTAGAGGATCCCC	acoR	474
		AGGGCGATGCCCCGGCCGATG	downstream	
	OLC58-R	CACATGGTCCTTCGAGTGTGC		
	OLC59-F	GCACACTCGAAGGACCATGTG	acoR	486
		CGCCGGCATGGCATCCGCATG	upstream	
	OLC59-R	ACCGAATTCGAGCTCGAGCCC		
		CTCGATCGCGCGGACGAACCA		
	OLC64-F	GGTCGACTCTAGAGGATCCCC	aco operon	654
		TAGTCGGCATCGCGCACCCGT	downstream	
	OLC64-R	GGAGAAATCGTCGGACGACGT		
	OLC65-F	ACGTCGTCCGACGATTTCTCCA	aco operon	708
		GTTGGTGAACAACAAGGAGC	upstream	
	OLC65-R	ACCGAATTCGAGCTCGAGCCC		
		ACAGCCTGACCACTTTCGTGC		

Table S3: Primers used in this study.

cclinical strains)Gene (PA O1)UniProtProductFunctionLog2 fold- changeAdjusted p- valueLog2 fold- changeAdjusted p- valueLask regulationfecl_2NANAP23484Putative RNA polymerase sigma factor FeclIron metabolism-2.674.02E-06-2.511.85E-04group_1318NANAP40883Regulatory protein PchRIron metabolism-3.573.88E-23-3.136.04E-25group_1539NANANAHypothetical proteinUnknown-2.963.51E-11-3.583.20E-13bauA_1PA0132bauAQ91700Beta-alanine-pyruvate aminotransferaseAminoacids (ILV) catabolism, propanoate metabolism3.151.00E-063.446.25E-39ActivationnirS_1PA0509nirkaQ91609Nitrite reductaseNitrogen metabolism-2.926.77E-10-2.933.30E-09RepressioncysG_2PA0510nirEaG3XD80Siroheme synthase NirENitrogen metabolism-3.008.64E-09-2.565.61E-06Repressiongroup_5012PA0512nirHaP95415NirHNitrogen metabolism-2.795.29E-06-2.359.60E-04group_3026PA0513nirGaP95414NirGNitrogen metabolism-2.795.29E-06-2.359.60E-04	Ref. 6 6 7 8,9 8,9 8,9 8,9 8,9 8,9 8 8 8 8 8 8 8 8
fecl_2NANAP23484Putative RNA polymerase sigma factor FeclIron metabolism-2.674.02E-06-2.511.85E-04group_1318NANAP40883Regulatory protein PchRIron metabolism-3.573.88E-23-3.136.04E-25group_1539NANANAHypothetical proteinUnknown-2.963.51E-11-3.583.20E-13bauA_1PA0132bauA·Q91700Beta-alanine-pyruvate aminotransferaseAminoacids (ILV) catabolism, propanoate metabolism3.151.00E-063.446.25E-39ActivationnirS_1PA0509nirNaQ91609Nitrie reductaseNitrogen metabolism-2.926.77E-10-2.933.30E-09RepressioncysG_2PA0510nirEaG3XD8Sinoheme synthase NirENitrogen metabolism-3.008.64E-09-2.565.61E-06Repressiongroup_3026PA0513nirGaP95415NirGNitrogen metabolism-3.278.64E-09-2.359.60E-04	6 6 7 8,9 8,9 8,9 8,9 8 8,9 8 8 8 8 8 8 8 8 8
group_1318NANAP40883Regulatory protein PcRIron metabolism-3.57 $3.88E-23$ $-3.13$ $6.04E-25$ group_1539NANANAHypothetical proteinUnknown-2.96 $3.51E-11$ $-3.58$ $3.20E-13$ bauA_1PA0132bauA $\cdot$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ <	6 7 8,9 8,9 8,9 8 8 8 8 8 8 8 8 8 8
group_1539NANAMy py othetical proteinUnknown-2.96 $3.51E-11$ $-3.58$ $3.20E-13$ bauA_1PA0132bauA $\cdot$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ <td>7 8,9 8,9 8,9 8 8 8 8 8 8 8 8 8</td>	7 8,9 8,9 8,9 8 8 8 8 8 8 8 8 8
bauA_1PA0132bauAQ91700Beta-alanine-pyruvate aminotransferaseAminoacids (ILV) catabolism, propanoate metabolism3.151.00E-063.446.25E-39ActivationnirS_1PA0509nirNaQ91609Nitrie reductaseNitrogen metabolism-2.926.77E-10-2.933.30E-09RepressioncysG_2PA0510nirEaG3XD80Siroheme synthase NirENitrogen, porphyrin and chlorophyll metabolism-3.008.64E-09-2.565.61E-06Repressiongroup_302PA0513nirGaP95415NirGNitrogen metabolism-3.278.64E-09-2.234.94E-04Repression	7 8,9 8,9 8,9 8 8 8 8 8 8 8 8
nirS_1PA0509nirNaQ91609Nitrite reductaseNitrogen metabolism-2.926.77E-10-2.933.30E-09RepressioncysG_2PA0510nirEaG3XD80Siroheme synthase NirENitrogen, porphyrin and chlorophyll metabolism-3.008.64E-09-2.565.61E-06Repressiongroup_5012PA0512nirHaP95415NirHNitrogen metabolism-3.278.64E-09-2.234.94E-04Repressiongroup_3026PA0513nirGaP95414NirGNitrogen metabolism-2.795.29E-06-2.359.60E-04	8,9 8,9 8 8 8 8 8 8 8 8 8
cysG_2PA0510nirEaG3XD80Siroheme synthase NireNitrogen, porphyrin and chlorophyll metabolism-3.008.64E-09-2.565.61E-06Repressiongroup_5012PA0512nirHaP95415NirHNitrogen metabolism-3.278.64E-09-2.234.94E-04Repressiongroup_3026PA0513nirGaP95414NirGNitrogen metabolism-2.795.29E-06-2.359.60E-04	8,9 8,9 8 8 8 8 8 8
group_3026       PA0512       nirH       a       P95415       NirH       Nitrogen metabolism       -3.27       8.64E-09       -2.23       4.94E-04       Repression         group_3026       PA0513       nirG       a       P95414       NirG       Nitrogen metabolism       -2.79       5.29E-06       -2.35       9.60E-04	8,9 8 8 8 8 8
group_3026 <b>PA0513</b> nirG a P95414 NirG Nitrogen metabolism -2.79 5.29E-06 -2.35 9.60E-04	8 8 8 8 8
	8 8 8 8
group_4848 PA0514 nirL a P95413 Heme d1 biosynthesis protein NirL Nitrogen metabolism -3.09 1.71E-08 -3.47 2.61E-08	8 8 8
group_3339 PA0515 nirD a P95412 Probable transcriptional regulator Nitrogen metabolism -2.99 2.04E-07 -2.03 1.30E-03	8 8
group_5595 <b>PA0516 nirF</b> a Q51480 Heme d1 biosynthesis Nitrogen metabolism -3.34 1.03E-10 -3.47 6.65E-16	8
nirS_2 <b>PA0519</b> nirS a P24474 Nitrite reductase Nitrogen metabolism -3.29 3.53E-07 -5.39 6.48E-51	
nirQ PA0520 nirQ b Q51481 Denitrification regulatory Nitrogen metabolism -4.27 3.41E-28 -4.51 3.08E-33 protein NirQ	8
qoxC         PA0521         nirO         b         G3XD44         Quinol oxidase subunit 3         Unknown         -4.65         2.60E-22         -4.60         4.38E-15	
group_6044 PA0522 nirP b Q51483 Hypothetical protein Unknown -2.90 1.59E-05 -2.99 7.07E-05	
norB PA0524 norB Q59647 Nitric oxide reductase subunit B Nitrogen metabolism -5.53 1.99E-23 -8.08 1.91E-79	8
group_3360 PA0672 hemO G3XCZ8 Heme oxygenase Porphyrin and chlorophyll -2.38 9.28E-05 -2.53 1.57E-17	
Phthiocerol/phenolphthiocer ppsC PA1137 PA1137 Q9I4J8 ol synthesis polyketide Unknown -4.31 1.74E-21 -3.55 1.62E-19 synthase	
rocR PA1196 ddaR Q9I4E2 Arginine utilization Aminoacids (arginine) 2.51 3.23E-05 3.22 5.81E-21	10
fecl_5 PA1300 PA1300 c Q9I444 sima factor Fecl Iron metabolism -3.17 3.51E-11 -2.50 2.25E-11	6
fecR_1 <b>PA1301 PA1301</b> c Q9I443 Protein FecR Iron metabolism -3.02 1.71E-08 -2.53 1.01E-13	6
ctpF PA1429 PA1429 Q9I3R5 Putative cation-transporting Unknown 2.89 8.45E-09 3.63 7.15E-35	
group_3703 PA1673 PA1673 Q9I352 Bacteriohemerythrin Unknown 2.16 1.57E-04 2.57 1.30E-14	
group_5526 PA1746 PA1746 Q9I2Z1 Hypothetical protein Unknown 2.42 1.59E-06 2.42 1.38E-09	
group_2540 PA1747 PA1747 Q9I2Z0 Hypothetical protein Unknown 2.57 8.64E-09 2.46 1.07E-08	
group_269 PA2033 PA2033 Q9I282 Hypothetical protein Unknown -2.24 1.65E-03 -2.84 7.11E-23	
ybdM <b>PA2126 cgrC</b> Q9I1Y9 CarC Unknown <b>2.60</b> 3.18E-07 <b>2.46</b> 1.05E-06	
group_6509 PA2321 gntK G3XD53 Gluconokinase Carbon metabolism (neptose phosphate) -2.73 4.03E-06 -2.41 2.68E-05 Repression	9
group_3203 PA2384 PA2384 Q9I195 Ferric uptake regulation Iron metabolism -2.44 1.09E-04 -2.56 8.25E-10	6
mbtH PA2412 PA2412 Q9I169 Hypothetical protein biosynthesis -2.12 1.66E-03 -2.27 6.19E-08	
group_3129 PA2468 fox/ Q9I114 Putative RNA polymerase Iron metabolism -2.32 1.63E-07 -2.03 1.07E-08	6
hmp PA2664 fhp Q9I0H4 Flavohemoprotein detaviliantian -2.41 1.85E-05 -2.38 1.86E-03	11
group_3982 PA2691 PA2691 Q9I0F1 NADH dehydrogenase-like Oxidative phosphorylation -4.38 1.03E-18 -3.45 3.32E-07	
hldD <b>PA3337 rfaD</b> Q9HYQ8 hortoge-6-primerase biocynthesis <b>3.40</b> 1.45E-12 <b>4.26</b> 1.62E-48	
yccM <b>PA3391 nosR</b> d Q9HYL3 Regulatory protein NosR Nitrogen metabolism -3.57 7.09E-14 -4.08 1.98E-30 Repression	8,9
nosZ PA3392 nosZ d Q9HYL2 Nitrous-oxide reductase Nitrogen metabolism -3.62 1.57E-10 -5.50 6.62E-51 Repression	8,9
nosD PA3393 nosD d Q9HYL1 Putative ABC transporter binding protein NosD Nitrogen metabolism -3.13 1.38E-10 -3.97 3.44E-20 Repression	8.9
nosY PA3395 nosY d Q9HYK9 permease protein NosY (nitrogen metabolism) -3.14 1.69E-07 -2.91 1.48E-06 Repression	2,2

nosL	PA3396	nosL	d	Q9HYK8	Copper-binding lipoprotein NosL	Nitrogen metabolism	-2.60	3.86E-05	-2.46	5.00E-05	Repression	8,9
group_3537	PA3411	PA3411		Q9HYJ4	Hypothetical protein	Unknown	-2.59	3.63E-06	-2.05	1.41E-03		
bfd	PA3530	bfd		Q9HY80	Bacterioferritin-associated ferredoxin	Unknown	-2.24	3.28E-10	-2.04	1.81E-10		
ykgO	PA3600	rpl36	е	Q9HY26	50S ribosomal protein L36 2	Ribosome structure	-2.99	6.77E-10	-2.87	3.60E-08		
rpmE2	PA3601	ykgM	е	Q9HY25	50S ribosomal protein L31 type B	Ribosome structure	-2.92	2.37E-10	-3.21	2.69E-26		
fdx_1	PA3809	fdx2	f	Q51383	2Fe-2S ferredoxin	Iron-sulfur protein	-2.72	4.17E-12	-3.02	7.98E-16		
hscA	PA3810	hscA	f	Q51382	Chaperone protein HscA	Protein stabilization	-2.56	4.41E-11	-2.60	6.55E-16		12
hscB	PA3811	hscB	f	Q9HXJ1	Co-chaperone protein HscB	Protein stabilization	-2.04	1.40E-06	-2.28	1.35E-14		12
iscA	PA3812	iscA	f	Q9HXJ0	Iron-binding protein IscA	[Fe-S] cluster biogenesis	-2.51	2.80E-10	-3.37	7.39E-26		13
iscU	PA3813	iscU	f	Q9HXI9	Iron-sulfur cluster assembly scaffold protein IscU	[Fe-S] cluster biogenesis	-2.40	3.46E-06	-3.34	1.95E-27		13
iscS_1	PA3814	iscS	f	Q9HXI8	Cysteine desulfurase IscS	Sulfur relay system, thiamine metabolism, [Fe- S] cluster biogenesis	-2.24	5.79E-05	-3.44	3.85E-29		13
iscR	PA3815	iscR	f	Q9HXI7	HTH-type transcriptional regulator IscR	[Fe-S] cluster biogenesis regulation	-2.42	4.44E-06	-3.65	4.38E-31		13
copA_4	PA3920	yvgX		Q9HX93	Copper-exporting P-type ATPase	Unknown	-2.28	3.75E-07	-2.63	4.15E-14		
ntaA	PA4155	PA4155		Q9HWM6	Nitrilotriacetate monooxygenase component A	Unknown	-3.09	5.86E-10	-2.23	6.24E-06		
fyuA_1	PA4156	fvbA		Q9HWM5	Pesticin receptor	Iron metabolism	-2.70	8.32E-12	-2.48	2.30E-09		14
fepC_1	PA4158	fepC		Q9HWM3	Ferric enterobactin transport ATP-binding protein FepC	ABC transporters (iron complex)	-2.30	6.59E-05	-2.21	3.50E-04		
fepB	PA4159	fepB		Q9HWM2	Ferrienterobactin-binding periplasmic protein	ABC transporters (iron complex)	-3.21	1.20E-10	-2.48	7.35E-06		
zupT	PA4467	PA4467	g	Q9HVV1	Zinc transporter ZupT	Unknown	-3.12	1.92E-08	-2.97	1.40E-15		
sodB_2	PA4468	sodM	g	P53652	Superoxide dismutase [Mn/Fe]	Oxydative and iron stress response	-3.17	1.19E-09	-3.09	4.37E-19		15
group_2514	PA4469	PA4469	g	Q9HVV0	Hypothetical protein	Unknown	-3.32	8.79E-09	-3.28	2.91E-23		
fumC_1	PA4470	fumC1	g	Q51404	Fumarate hydratase class II	Carbon metabolism (cytrate circle), iron stress response	-3.14	6.08E-08	-3.16	2.75E-18		15
group_4225	PA4471	fagA	g	G3XD99	Hypothetical protein	Unknown	-3.52	3.14E-17	-3.23	1.64E-25		
group_3398	PA4570	PA4570		Q9HVL4	Hypothetical protein	Unknown	-4.12	3.14E-17	-4.32	4.87E-31		
ссрА	PA4587	ccpR		P14532	Cytochrome c551 peroxidase	Oxydative stress response	3.16	3.49E-11	3.27	6.90E-16	Repression	7,16
group_1579	PA4625	cdrA		Q9HVG6	Cyclic diguanylate-regulated TPS partner A, CdrA	Adhesion and biofilm matrix structure	2.53	8.83E-05	2.06	6.78E-10		17
fecl_3	PA4896	PA4896		Q9HUR7	Putative RNA polymerase sigma factor Fecl	Iron metabolism	-3.04	6.78E-10	-2.23	1.49E-08		6
group_4196	PA5027	PA5027		Q9HUE2	Hypothetical protein	Unknown	2.35	7.70E-05	2.82	2.70E-20	Activation	9
arcD	PA5170	arcD		P18275	Arginine/ornithine antiporter	Aminoacids (arginine) metabolism	2.36	4.52E-04	2.49	5.13E-04		10
NA	PA5369.3	PA5369	.3	NA	tRNA-Ala	Aminoacyl-tRNA biosynthesis	2.12	7.09E-03	4.50	3.69E-21		
pka	PA5475	PA5475		Q9HT95	Protein lysine acetyltransferase Pka	Unknown	2.04	4.85E-04	2.30	1.81E-10		

Table S4: List of *P. aeruginosa* genes differentially expressed in presence of *S. aureus* in the context of a competitive interaction. PA2596 competition strain was cultivated in absence or presence of SA2599 or SA2597. RNAs were extracted after 4 hours of culture and RNAseq analysis was performed as described in material and methods. A gene was considered as differentially expressed when the Fold Change (FC) was > |2log<sub>2</sub>| with an adjusted *P*-value<0.05 in presence of both SA strains. Genes from the same operon are annotated with an identical letter. Grey cells indicate genes that were also dysregulated in the context of coexistence (Table S5). Functional classification was performed thanks to KEGG database and literature.

Gene (clinical							SA2599/PA2600		SA2597/PA2600		LasR	
strains)	Gene (PAC	D1)		UniProt	Product	Function	Log <sub>2</sub> fold- change	Adjusted p- value	Log <sub>2</sub> fold- change	Adjusted p- value	regulation	Ref.
group_955	NA	NA		NA	Hypothetical protein	Unknown	-2.20	3.84E-09	-2.56	1.54E-05		
eamB_1	NA	NA		P38101	Cysteine/O-acetylserine efflux protein	Aminoacids transport	2.04	5.37E-04	2.31	2.69E-03		
group_511	NA	NA		NA	Hypothetical protein	Unknown	-2.04	2.34E-03	-2.77	5.28E-03		
bauD	PA0129	bauD		Q91703	Putative GABA permease	Aminoacids (β-alanine) catabolism	2.90	1.53E-15	3.07	2.83E-14		18
bauB	PA0131	bauB		Q9I701	Beta-alanine degradation protein BauB	Aminoacids (β-alanine) catabolism	2.27	2.30E-05	2.61	2.31E-04		18
cysG_2	PA0510	nirE	а	G3XD80	Siroheme synthase NirE	Nitrogen, porphyrin and chlorophyll metabolism	-2.42	6.73E-05	-2.72	1.36E-03	Repression	8,9
group_3026	PA0513	nirG	а	P95414	NirG	Nitrogen metabolism	-2.69	3.29E-05	-2.97	2.87E-03		8
group_3339	PA0515	nirD	а	P95412	Probable transcriptional regulator	Nitrogen metabolism	-2.62	1.99E-03	-3.37	3.73E-04		8
group_5595	PA0516	nirF	а	Q51480	Heme d1 biosynthesis protein NirF	Nitrogen metabolism	-3.53	1.60E-08	-3.88	1.44E-06		8
nirM	PA0518	nirM	а	P00099	Cytochrome c-551	Nitrogen metabolism	-2.62	4.27E-03	-3.27	3.88E-03		8
nirS_2	PA0519	nirS	а	P24474	Nitrite reductase	Nitrogen metabolism	-3.27	7.25E-06	-4.43	2.95E-08		8
nirQ	PA0520	nirQ		Q51481	Denitrification regulatory protein NirQ	Nitrogen metabolism	-3.25	7.06E-05	-4.12	2.10E-06		8
norC	PA0523	norC	h	Q59646	Nitric oxide reductase subunit C	Nitrogen metabolism	-3.59	2.99E-05	-6.23	4.50E-14		8
norB	PA0524	norB	h	Q59647	Nitric oxide reductase subunit B	Nitrogen metabolism	-5.82	3.20E-18	-5.77	2.52E-12		8
group_4191	PA0525	norD	h	Q51484	Probable dinitrification protein NorD	Nitrogen metabolism	-3.86	1.68E-06	-4.82	1.23E-08		8
group_364	PA0526	PA0526	6	G3XDA0	Hypothetical protein	Unknown	-3.38	6.53E-05	-4.60	3.45E-06		
NA	PA0668.3	NA		NA	tRNA-Ala(tgc)	Aminoacyl-tRNA biosynthesis	3.45	5.66E-05	7.57	1.86E-22		
acrC_2	PA0746	PA0746	6 i	Q91513	Acryloyl-CoA reductase (NADH)	Unknown	2.80	1.01E-12	3.12	2.02E-15		
mmsA_2	PA0747	PA0747	7 i	Q91512	Methylmalonate- semialdehyde dehydrogenase	Aminoacids (isoleucine, leucine, valine) catabolism, propanoate and carbon metabolism	2.08	1.04E-05	2.31	1.44E-06		
acnM	PA0794	PA0794	ţ	Q9I5E4	Aconitate hydratase A	Propanoate metabolism	2.18	5.28E-10	2.90	1.80E-07		
aroP_2	PA0866	aroP2		Q9I575	Aromatic amino acid transport protein AroP	Unknown	2.30	8.73E-06	2.74	1.02E-03		
acs_1	PA0887	acsA		Q9I558	Acetyl-coenzyme A synthetase	Propanoate, carbon and pyruvate metabolism	3.08	5.58E-10	3.27	2.24E-07	Repression	7
yybH	PA1325	ууЬН	j	Q9I419	Putative protein YybH	Unknown	2.16	1.03E-03	2.73	1.94E-05		
ilvA_1	PA1326	ilvA	j	Q9l418	L-threonine dehydratase biosynthetic IIvA	Carbon metabolism, aminoacids (isoleucine, leucine, valine) biosynthesis	2.19	4.61E-05	2.46	6.93E-04		
group_1804	PA1874	PA1874	<b>k</b>	Q9I2M3	Hypothetical protein	Antibiotic resistance, quorum sensing	-2.54	5.92E-05	-2.68	2.83E-04	Activation	9,19
group_4895	PA1875	PA1875	5 k	Q9I2M2	Hypothetical protein	Antibiotic resistance	-2.94	1.72E-09	-2.80	1.14E-05	Activation	9,19
prsE_3	PA1877	PA1877	<b>r</b> k	Q9I2M0	I ype I secretion system membrane fusion protein	Antibiotic resistance	-2.29	8.13E-03	-3.04	1.51E-07		19
group_6609	PA1878	PA1878	3	Q9I2L9	Hypothetical protein	Unknown	-2.03	2.47E-05	-2.38	1.01E-03		
group_2034	PA1914	hvn		Q912J0	Hypothetical protein	Unknown	-3.87	9.10E-16	-4.61	3.05E-20	Activation	7
degU_4	PA1978	erbR		P29369	Transcriptional regulatory protein DegU	Ethanol stress response	2.28	2.43E-05	3.06	1.45E-07	Repression	9,20
luxQ	PA1992	ercS		Q9I2B7	Autoinducer 2 sensor kinase/phosphatase LuxQ	Ethanol stress response	2.13	9.50E-08	2.11	2.65E-04		20
accA1_2	PA2012	liuD	I	Q91299	Acetyl-/propionyl-coenzyme A carboxylase alpha chain	Aminoacids (leucine) and monoterpenes catabolism	2.30	7.81E-07	2.69	2.15E-06		21
menB	PA2013	liuC	I	Q91298	1%2C4-dihydroxy-2- naphthoyl-CoA synthase	Aminoacids (leucine) and monoterpenes catabolism	3.02	1.20E-11	3.29	7.09E-12		21
group_2911	PA2014	liuB	I	Q91297	Methylmalonyl-CoA carboxyltransferase 12S subunit	Aminoacids (leucine) and monoterpenes catabolism	2.61	1.20E-08	2.85	4.87E-10	Activation	9,21
mmgC_7	PA2015	liuA	I	Q91296	Acyl-CoA dehydrogenase	Aminoacids (leucine) and monoterpenes catabolism	2.62	3.07E-08	2.76	5.26E-09		21
cueR_2	PA2016	liuR		Q91295	HTH-type transcriptional regulator CueR	Aminoacids (leucine) and monoterpenes catabolism	2.61	4.02E-08	3.17	4.01E-06		21

group_13	PA2040	pauA4	Q	291275	Gamma-glutamylputrescine synthetase PuuA	Polyamines catabolism, aminoacids (glutamine) biosynthesis	2.36	2.02E-08	2.77	8.62E-09		22
kynU	PA2080	kynU	Q	291235	Kynureninase KynU	Aminoacids (tryptophan) catabolism	2.63	9.29E-10	2.67	7.28E-07	Activation	7
group_1851	PA2166	PA2166	Q	Q9I1U9	Hypothetical protein	Unknown	-2.23	5.77E-05	-2.82	1.56E-04	Activation	7
gntR_3	PA2320	gntR	Q	Q9I1F6	HTH-type transcriptional regulator GntR	Carbon metabolism (pentose phosphate)	-2.63	9.55E-11	-2.71	1.51E-05		
group_6509	PA2321	gntK	G	G3XD53	Gluconokinase GntK	Carbon metabolism (pentose phosphate)	-3.67	1.18E-14	-3.42	5.37E-06	Repression	9,23
group_1686	PA2462	PA2462	Q	291120	Hypothetical protein	Unknown	2.36	1.33E-10	2.34	7.08E-11		
tsdA	PA2481	PA2481	m Q	29 101	Thiosulfate dehydrogenase	Unknown	2.68	1.47E-07	2.09	1.59E-04		
group_3818	PA2482	PA2482	m Q	291100	Cytochrome c4	Unknown	2.73	8.24E-08	2.39	6.15E-04		
mmgC_5	PA2552	acdB	n Q	Q9I0T2	Acyl-CoA dehydrogenase	Unknown	2.49	6.18E-12	2.82	2.35E-10	Activation	9
thIA_1	PA2553	PA2553	n Q	Q9I0T1	Acetyl-CoA acetyltransferase	Carbon and fatty acids metabolism	2.46	7.14E-11	2.72	1.52E-08	Activation	9
group_5789	PA2554	PA2554	n Q	Q9I0T0	Putative oxidoreductase	Unknown	2.78	1.75E-13	3.05	1.43E-09	Activation	9
acsA_1	PA2555	PA2555	n Q	2910S9	Acetyl-coenzyme A synthetase	Propanoate, carbon and pyruvate metabolism	2.83	1.51E-10	2.93	8.32E-07	Activation	9
fadD3	PA2557	PA2557	Q	Q9I0S7	3-[(3aS%2C4S%2C7aS)-7a methyl-1%2C5-dioxo- octahydro-1H-inden	Unknown	3.04	2.30E-14	3.16	2.62E-11		
lecA	PA2570	lecA	Q	205097	PA-I galactophilic lectin LecA	Adhesion, biofilm formation	-3.40	1.59E-12	-2.61	1.13E-03	Activation	7,9,24
group_5847	PA2662	PA2662	o Q	2910H6	Hypothetical protein	Unknown	-3.18	1.66E-07	-3.57	3.71E-05		
group_3164	PA2663	ppyR	o Q	2910H5	PsI and pyoverdine operon regulator, PpyR	Iron metabolism, biofilm formation and virulence	-2.83	7.91E-04	-4.04	9.15E-07		25
hmp	PA2664	fhp	Q	Q910H4	Flavohemoprotein	Iron metabolism, NO detoxification	-5.40	1.82E-18	-5.66	3.69E-14		11
lip_2	PA2862	lipA	P	P26876	Triacylglycerol lipase	Glycerolipid metabolism, virulence (lipase activity)	2.31	6.63E-05	2.47	4.68E-04		
group_2901	PA3038	opdQ	Q	29HZH0	Porin-like protein NicP	Membrane transports (in response to stress)	2.75	8.42E-07	2.98	2.71E-06	Repression	7,26
ydfJ	PA3079	PA3079	p Q	Q9HZC9	Membrane protein YdfJ	Unknown	2.43	2.32E-07	2.29	2.55E-06		
group_6150	PA3080	PA3080	p Q	29HZC8	Ycf48-like protein	Unknown	2.33	5.77E-09	2.24	1.34E-04		
eda_2	PA3181	edaA	q O	D68283	2-dehydro-3-deoxy- phosphogluconate aldolase	Carbon metabolism (pentose phosphate)	-2.85	2.30E-14	-3.17	6.61E-11	Activation	7
pgl_1	PA3182	pgl	q Q	Q9X2N2	6-phosphogluconolactonase	Carbon metabolism (pentose phosphate)	-3.10	1.45E-13	-3.13	1.21E-08	Activation	7
zwf_2	PA3183	zwf	q O	D68282	Glucose-6-phosphate 1- dehydrogenase	Carbon metabolism (pentose phosphate)	-2.77	4.39E-08	-3.05	4.13E-11	Activation	7
ugpC	PA3187	gltK	r Q	Q9HZ51	Sn-glycerol-3-phosphate import ATP-binding protein UgpC	ABC transporter (oligosaccharides, polyol, lipids and monosaccharides)	-3.88	1.55E-18	-3.03	4.95E-08		
ugpA	PA3189	gltF	r Q	Q9HZ49	Sn-glycerol-3-phosphate	ABC transporter	-2.66	7.46E-08	-2.25	4.99E-03	Activation	7
group 58/2	PA3100	altB	0	704748	Putative sugar-binding	Carbon metabolism	-5 31	1 /3E-50	-4.96	2 08E-23	Activation	7
edd	PA3194	edd	P	231961	periplasmic protein Phosphogluconate	(pentose phosphate) Carbon metabolism	-2 16	1 26E-04	-2 55	2 31E-10	Activation	7
epd 1	PA3195	gapA	P	27726	dehydratase D-erythrose-4-phosphate	(pentose phosphate) Carbon metabolism	-2.27	1.15E-05	-2.42	1.87E-07	Activation	7
aroup 5870	PA3233	PA3233	Q	29HZ07	dehydrogenase Hypothetical protein	(pentose phosphate) Unknown	2.04	1.84E-08	2.35	1.90E-05	Repression	7
actP 1	PA3234	vicG	s Q	29HZ06	Cation/acetate symporter	Unknown	2.86	3.07E-07	2.89	5.65E-05	Repression	7
vicH 1	PA3235	vicH	s Q	29HZ05	ActP Inner membrane protein	Unknown	2.09	4.22E-04	2.31	6.97E-05	Repression	7
nosL	PA3396	nosL	Q	29НҮК8	Copper-binding lipoprotein	Nitrogen metabolism	-2.63	2.89E-04	-3.00	7.77E-03	Repression	9
mmsB	PA3569	mmsB	t P.	P28811	NosL 3-hydroxyisobutyrate	Aminoacids (ILV) catabolism	2.30	2.60E-10	3.27	1.43E-09		
A -	B4	_			Methylmalonate-	Aminoacids (ILV)		0.007		0 7-7		
mmsA_1	PA3570	mmsA	τΡ	-28810	semialdenyde dehydrogenase	catabolism, propanoate and carbon metabolism	2.32	8.63E-09	3.32	3.75E-08		
iscR	PA3815	iscR	Q	Q9HXI7	HTH-type transcriptional regulator IscR	isc operon regulation	-2.41	2.64E-06	-2.10	3.56E-03		13
dauA	PA3863	dauA	Q	Q9HXE3	FAD-dependent catabolic D- arginine dehydrogenase DauA	Aminoacids (arginine, ornithine) catabolism	2.12	6.53E-05	2.10	3.23E-03		
argT_1	PA3865	PA3865	Q	Q9HXE1	Lysine/arginine/ornithine- binding periplasmic protein	ABC transporter (arginine, ornithine)	2.47	2.45E-05	2.54	2.08E-04		

group_3022	PA3922	PA3922	u	Q9HX91	Hypothetical protein	Unknown	2.18	3.16E-07	2.13	8.05E-11		
group_4203	PA3923	PA3923	u	Q9HX90	Hypothetical protein	Unknown	2.39	2.29E-07	2.38	6.61E-11	Activation	9
group_2095	PA4022	hdhA		Q9HX05	Hypothetical protein	Pyruvate and carbon metabolism, hydrazone utilization	2.35	8.00E-12	2.50	4.71E-07		27
yhdG_3	PA4023	eutP		Q9HX04	Putative amino acid permease YhdG	Unknown	2.79	7.48E-07	3.33	4.81E-08		
acoR_1	PA4147	acoR		Q9HWN4	Acetoin catabolism regulatory protein	Unknown	2.02	1.07E-05	2.26	1.51E-05		
fabG_10	PA4148	PA4148	v	Q9HWN3	3-oxoacyl-[acyl-carrier- protein] reductase FabG	Butanoate metabolism	2.52	7.65E-03	4.20	2.95E-08		
acoA	PA4150	acoA	v	Q9HWN1	Acetoin:2%2C6- dichlorophenolindophenol oxidoreductase subunit a	Unknown	2.42	6.84E-03	3.89	5.15E-16		
acoB	PA4151	acoB	v	Q9HWN0	Acetoin:2%2C6- ) dichlorophenolindophenol oxidoreductase subunit b	Unknown	2.95	8.68E-07	3.51	3.12E-15		
acoC	PA4152	acoC	v	Q9HWM9	Dihydrolipoyllysine-residue acetyltransferase component of acetoin cleaving system	Carbon and pyruvate metabolism	3.00	4.18E-04	4.89	3.22E-19		
ydjJ_2	PA4153	PA4153	v	Q9HWM8	2,3-butanediol dehydrogenase	Butanoate metabolism	2.33	9.28E-03	3.94	1.07E-13		
pchA_2	PA4231	pchA		Q51508	Salicylate biosynthesis isochorismate synthase	Ubiquinone and non- ribosomal siderophore peptides synthesis	-2.72	3.55E-05	-2.18	5.58E-03		
cckA	PA4293	pprA	w	Q9HWA7	Sensor kinase CckA	Membrane permeability	-2.54	2.98E-06	-3.28	1.55E-08	Activation	9,28
group_719	PA4294	PA4294	w	Q9HWA6	Hypothetical protein	Unknown	-3.35	9.82E-14	-5.10	7.29E-14	Activation	9
group_2484	PA4300	tadC	x	Q9HWA0	TadC	Flp pilus assembly	-2.45	8.38E-06	-2.73	4.22E-05	Activation	7.9
group_6095	PA4301	tadB	x	Q9HW99	TadB	Flp pilus assembly	-2.47	1.84E-06	-2.73	1.06E-04		31
group_4498	PA4302	tadA	x	Q9HW98	TadA ATPase	Flp pilus assembly	-2.07	3.10E-05	-2.99	4.73E-06	Activation	7,9,29
outD	PA4304	rcpA	x	Q9HW96	RcpA	Flp pilus assembly	-2.48	2.53E-07	-2.92	1.79E-08	Activation	7,9,29
group_95	PA4306	flp		Q9HW94	Type IVb pilin, Flp	Flp pilus assembly	-2.94	1.73E-05	-2.67	1.65E-03	Activation	7,9,29
group_1582	PA4638	PA4638	•	Q9HVF3	Hypothetical protein	Unknown	-2.33	3.36E-08	-2.42	1.55E-04		
yabJ_1	PA5083	dguB	у	Q9HUA0	2-iminobutanoate/2- iminopropanoate deaminase	Aminoacids (glutamine) metabolism	2.70	1.04E-04	3.77	1.29E-09		30
dadA1_1	PA5084	dguA	у	Q9HU99	D-amino acid dehydrogenase 1	Aminoacids (glutamine, phenylalanine) metabolism	2.70	7.78E-05	4.01	2.78E-08		30
group_5220	PA5096	PA5096		Q9HU87	Glycine betaine-binding periplasmic protein OusX	ABC transporter (glycine, betaine, proline)	2.51	2.24E-10	2.54	7.31E-06		
proY_1	PA5097	hutT	z	Q9HU86	Proline-specific permease ProY	Aminoacids (histidine) catabolism	2.46	1.71E-09	2.37	3.88E-07		31
hutH_1	PA5098	hutH	z	Q9HU85	Histidine ammonia-lyase	Aminoacids (histidine) catabolism	3.16	3.35E-16	2.95	2.39E-12		31
pucl_1	PA5099	PA5099	z	Q9HU84	Putative allantoin permease	Aminoacids (histidine) catabolism	2.84	6.41E-08	2.07	1.95E-05		31
hutU	PA5100	hutU		Q9HU83	Urocanate hydratase	Aminoacids (histidine) catabolism	2.65	4.00E-06	2.19	2.65E-08		31
artJ	PA5153	PA5153	1	Q9HU31	ABC transporter arginine- binding protein 1	ABC transporter (arginine)	2.06	3.96E-05	2.23	8.34E-13		
NA	PA5160.1	PA5160	.1	NA	tRNA-Thr(tgt)	Aminoacyl-tRNA biosynthesis	3.26	5.20E-08	6.82	3.05E-20		
group_4368	PA5383	yeiH		Q9HTI1	Hypothetical protein	Unknown	-2.93	3.32E-06	-3.25	1.16E-03		
group_3039	PA5460	PA5460	,	Q9HTB0	Hypothetical protein	Unknown	-2.24	7.66E-09	-2.23	4.96E-03		
group_5371	PA5469	PA5469	,	Q9HTA1	Hypothetical protein	Unknown	2.26	1.56E-03	3.02	4.99E-04		

Table S5: List of *P. aeruginosa* genes differentially expressed in presence of *S. aureus* in the context of coexistence. PA2600 coexistence strain was cultivated in the absence or presence of SA2599 or SA2597. RNAs were extracted after 4 hours of culture and a RNAseq analysis was performed as described in material and methods. A gene was considered as differentially expressed when the Fold Change (FC) was >  $|2log_2|$  with an adjusted *P*-value<0.05 in presence of both SA strains. Genes from the same operon are annotated with an identical letter. Grey cells indicate genes that were also dysregulated in competition couples (Table S4). Functional classification was performed thanks to KEGG database and literature.



#### Figure S1: Schematic representation of the employed methodology.

**A.** Determination of interaction state within *S. aureus-P. aeruginosa* co-isolated pairs. Pairs of strains are co-isolated from a single sputum sample. Interaction state is tested during plate and liquid tests, as described in materials and methods and by Briaud *et al.* (3). Results of competition tests (pictures and kinetics) were obtained for a previous study (3).

**B. Strain pairs used in transcriptomic analyses.** Pairs SA2597/PA2596 and SA2599/PA2600 were isolated from two different patients. Interaction state of crossed pairs was determined as above and confirmed that it is solely led by *P. aeruginosa* (3). The 21 strain pairs used for qRT-PCR confirmation were both isolated from different patients, except in one case (Table S1).



Figure S2: *P. aeruginosa* metabolic pathways and associated genes up-regulated (red) or downregulated (green) in coexistence with *S. aureus*. PA2600 coexistence strain was cultivated in absence or presence of SA2599 or SA2597. RNAs were extracted after 4 hours of culture and RNAseq analysis was performed. A gene was considered as differentially expressed when the Fold Change (FC) was > |2log<sub>2</sub>| with an adjusted *P*-value<0.05. Genes from the same operon are annotated with an identical letter. Genes tested in RT-qPCR and confirmed for PA2600 are underlined. Functional classification and pathway constructions were performed thanks to KEGG database and literature.



Figure S3: Acetoin concentration in supernatant of *S. aureus* SA2599, *B. subtilis*, *B. cenocepacia* and *S. maltophilia* monocultures (grey bars) or co-cultures with *P. aeruginosa* PA2600 (black bars). Acetoin was quantified from supernatant after 4h of culture. Bars represent the mean acetoin concentration + SEM from three independent experiments.



SA2599 📈 SA2599 supernatant 🔳 SA UAMS-1 WT supernatant

Figure S4: Fold change of *P. aeruginosa acoR*, *PA4148*, *liuA* and *zwf* gene expression induced by culture with *S. aureus* (grey bars) or its supernatant (hatched and black bars). *P. aeruginosa* PA2600 strain was cultivated in the absence or presence of *S. aureus* SA2599 or filtered supernatant of *S. aureus* SA2599 and UAMS-1 WT. RNAs were extracted after 4 hours of culture and gene expression was assayed by RT-qPCR. Bars represent the mean fold change + SEM from three independent experiments. Dot lines indicate a fold change = |2|. \**P*<sub>adj</sub><0.05, \*\*\*\**P*<sub>adj</sub><0.0001 ANOVA with Tukey's correction.



Figure S5: Monitoring of acetoin concentration in *S. aureus* and *P. aeruginosa* monocultures or coculture (A,B) or in *S. aureus* supernatant inoculated with *P. aeruginosa* (B,C), for the pairs SA146/PA146 (A,C) and SA153/PA153A (B,D).

**A**, **B**. *S. aureus* and *P. aeruginosa* were cultivated in monoculture or co-culture. Acetoin was quantified from supernatant each hour. Points represent the mean acetoin concentration  $\pm$  SEM from two independent experiments per pair.

**C**, **D**. A 4-hour filtered supernatant of *S. aureus* was inoculated with *P. aeruginosa* culture or sterile medium for controls. Acetoin was quantified from supernatant each hour. Points represent the mean acetoin  $\pm$  SEM from three independent experiments per pair.



**Figure S6: Acetoin concentration in CF sputa from patients.** Sputa from *S. aureus* mono-infected patients (n=9) or *S. aureus* and *P. aeruginosa* co-infected patients (n=11) were gathered and acetoin concentration was quantified. Bars represent the median acetoin concentration normalized on *S. aureus* concentration in each sputum.



Figure S7: Acetoin concentration in cultures of *S. aureus* strains from competition and coexistence couples. Each *S. aureus* strain from competition (n=11) and coexistence (n=12) couples was cultivated for 6 hours in BHI and acetoin was dosed from supernatant. Bars represent the median acetoin concentration  $\pm$  95% CI. ns *P*>0.05 Mann-Whitney test.



# Figure S8: Glucose concentrations in cultures of *S. aureus* (A) and *P. aeruginosa* (B) strains from competition and coexistence pairs.

**A.** Each *S. aureus* strain from competition (n=12) and coexistence (n=12) couples was cultivated in *P. aeruginosa* PA2600 filtered supernatant for 6 hours and glucose was quantified from supernatant. No glucose was detected. Dotted line indicates the initial glucose concentration in *P. aeruginosa* supernatant.

**B.** Each *P. aeruginosa* strain from competition (n=12) and coexistence (n=12) couples was cultivated in *S. aureus* SA2599 filtered supernatant for 4 hours and glucose was quantified from supernatant. Bars represent the median glucose concentration  $\pm$  95% CI. Dotted line indicates the initial glucose concentration in *S. aureus* supernatant. ns *P*>0.05 Mann-Whitney test.



**Figure S9: Growth kinetic of** *S. aureus* **cultivated in absence or presence of acetoin.** SA2599 was cultivated during 24h in the absence of acetoin or in the presence of acetoin in different proportions ranging from 0.2µM to 6000µM per 10<sup>6</sup> *S. aureus*. Lines represent the mean optical density of three technical replicates.

#### Supplementary data references

(1-5)(6-10)(11-15)(16-20)(21-25)(26-31)

- Carriel D, Simon Garcia P, Castelli F, Lamourette P, Fenaille F, Brochier-Armanet C, Elsen S, Gutsche I. 2018. A Novel Subfamily of Bacterial AAT-Fold Basic Amino Acid Decarboxylases and Functional Characterization of Its First Representative: Pseudomonas aeruginosa LdcA. Genome Biol Evol 10:3058–3075.
- 2. Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ. 2005. ExsE, a secreted regulator of type III secretion genes in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 102:8006–8011.
- 3. Briaud P, Camus L, Bastien S, Doléans-Jordheim A, Vandenesch F, Moreau K. 2019. Coexistence with Pseudomonas aeruginosa alters Staphylococcus aureus transcriptome, antibiotic resistance and internalization into epithelial cells. Sci Rep 9.
- 4. Tsang LH, Cassat JE, Shaw LN, Beenken KE, Smeltzer MS. 2008. Factors Contributing to the Biofilm-Deficient Phenotype of Staphylococcus aureus sarA Mutants. PLoS ONE 3:e3361.
- 5. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76:1648–1652.
- Chevalier S, Bouffartigues E, Bodilis J, Maillot O, Lesouhaitier O, Feuilloley MGJ, Orange N, Dufour A, Cornelis P. 2017. Structure, function and regulation of Pseudomonas aeruginosa porins. FEMS Microbiol Rev 41:698–722.
- Schuster M, Lostroh CP, Ogi T, Greenberg EP. 2003. Identification, timing, and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis. J Bacteriol 185:2066– 2079.
- Borrero-de Acuña JM, Rohde M, Wissing J, Jänsch L, Schobert M, Molinari G, Timmis KN, Jahn M, Jahn D. 2016. Protein Network of the Pseudomonas aeruginosa Denitrification Apparatus. J Bacteriol 198:1401–1413.
- 9. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. 2003. Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment. J Bacteriol 185:2080–2095.
- 10. Lundgren BR, Sarwar Z, Pinto A, Ganley JG, Nomura CT. 2016. Ethanolamine Catabolism in Pseudomonas aeruginosa PAO1 Is Regulated by the Enhancer-Binding Protein EatR (PA4021) and the Alternative Sigma Factor RpoN. J Bacteriol 198:2318–2329.
- 11. Arai H, Hayashi M, Kuroi A, Ishii M, Igarashi Y. 2005. Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of Pseudomonas aeruginosa. J Bacteriol 187:3960–3968.
- 12. Campos-García J, G Ordóñez L, Soberón-Chávez G. 2000. The Pseudomonas aeruginosa hscA gene encodes Hsc66, a DnaK homologue. Microbiology (Reading, Engl) 146 (Pt 6):1429–1435.
- Romsang A, Duang-Nkern J, Leesukon P, Saninjuk K, Vattanaviboon P, Mongkolsuk S. 2014. The ironsulphur cluster biosynthesis regulator IscR contributes to iron homeostasis and resistance to oxidants in Pseudomonas aeruginosa. PLoS ONE 9:e86763.
- 14. Elias S, Degtyar E, Banin E. 2011. FvbA is required for vibriobactin utilization in Pseudomonas aeruginosa. Microbiology (Reading, Engl) 157:2172–2180.

- 15. Hassett DJ, Howell ML, Ochsner UA, Vasil ML, Johnson Z, Dean GE. 1997. An operon containing fumC and sodA encoding fumarase C and manganese superoxide dismutase is controlled by the ferric uptake regulator in Pseudomonas aeruginosa: fur mutants produce elevated alginate levels. J Bacteriol 179:1452–1459.
- 16. Eichner A, Günther N, Arnold M, Schobert M, Heesemann J, Hogardt M. 2014. Marker genes for the metabolic adaptation of Pseudomonas aeruginosa to the hypoxic cystic fibrosis lung environment. Int J Med Microbiol 304:1050–1061.
- 17. Borlee BR, Goldman AD, Murakami K, Samudrala R, Wozniak DJ, Parsek MR. 2010. Pseudomonas aeruginosa uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. Mol Microbiol 75:827–842.
- Yao X, He W, Lu C-D. 2011. Functional characterization of seven γ-Glutamylpolyamine synthetase genes and the bauRABCD locus for polyamine and β-Alanine utilization in Pseudomonas aeruginosa PAO1. J Bacteriol 193:3923–3930.
- 19. Zhang L, Mah T-F. 2008. Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. J Bacteriol 190:4447–4452.
- 20. Mern DS, Ha S-W, Khodaverdi V, Gliese N, Görisch H. 2010. A complex regulatory network controls aerobic ethanol oxidation in Pseudomonas aeruginosa: indication of four levels of sensor kinases and response regulators. Microbiology (Reading, Engl) 156:1505–1516.
- 21. Aguilar JA, Zavala AN, Díaz-Pérez C, Cervantes C, Díaz-Pérez AL, Campos-García J. 2006. The atu and liu clusters are involved in the catabolic pathways for acyclic monoterpenes and leucine in Pseudomonas aeruginosa. Appl Environ Microbiol 72:2070–2079.
- Chou HT, Li J-Y, Peng Y-C, Lu C-D. 2013. Molecular characterization of PauR and its role in control of putrescine and cadaverine catabolism through the γ-glutamylation pathway in Pseudomonas aeruginosa PAO1. J Bacteriol 195:3906–3913.
- 23. Daddaoua A, Corral-Lugo A, Ramos J-L, Krell T. 2017. Identification of GntR as regulator of the glucose metabolism in Pseudomonas aeruginosa. Environ Microbiol 19:3721–3733.
- Worstell NC, Singla A, Saenkham P, Galbadage T, Sule P, Lee D, Mohr A, Kwon JS-I, Cirillo JD, Wu H-J. 2018. Hetero-Multivalency of Pseudomonas aeruginosa Lectin LecA Binding to Model Membranes. Sci Rep 8:8419.
- 25. Attila C, Ueda A, Wood TK. 2008. PA2663 (PpyR) increases biofilm formation in Pseudomonas aeruginosa PAO1 through the psl operon and stimulates virulence and quorum-sensing phenotypes. Appl Microbiol Biotechnol 78:293–307.
- 26. Fowler RC, Hanson ND. 2015. The OpdQ porin of Pseudomonas aeruginosa is regulated by environmental signals associated with cystic fibrosis including nitrate-induced regulation involving the NarXL two-component system. Microbiologyopen 4:967–982.
- Taniyama K, Itoh H, Takuwa A, Sasaki Y, Yajima S, Toyofuku M, Nomura N, Takaya N. 2012. Group X aldehyde dehydrogenases of Pseudomonas aeruginosa PAO1 degrade hydrazones. J Bacteriol 194:1447–1456.
- 28. Wang Y, Ha U, Zeng L, Jin S. 2003. Regulation of membrane permeability by a two-component regulatory system in Pseudomonas aeruginosa. Antimicrob Agents Chemother 47:95–101.

- 29. Bernard CS, Bordi C, Termine E, Filloux A, de Bentzmann S. 2009. Organization and PprB-dependent control of the Pseudomonas aeruginosa tad Locus, involved in Flp pilus biology. J Bacteriol 191:1961–1973.
- 30. He W, Li G, Yang C-K, Lu C-D. 2014. Functional characterization of the dguRABC locus for D-Glu and d-Gln utilization in Pseudomonas aeruginosa PAO1. Microbiology (Reading, Engl) 160:2331–2340.
- 31. Gerth ML, Ferla MP, Rainey PB. 2012. The origin and ecological significance of multiple branches for histidine utilization in Pseudomonas aeruginosa PAO1. Environ Microbiol 14:1929–1940.