

The Long Hunt for pssR -Looking for a Phospholipid Synthesis Transcriptional Regulator, Finding the Ribosome

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| 1 | The Long Hunt For $pssR$ – looking for a phospholipid synthesis transcriptional |
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| 2 | regulator, finding the ribosome. |
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| 4 | Running title : <i>pssR1</i> phenotype due to an anti-SD sequence mutation |
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15 Abstract

The phospholipid (PL) composition of bacterial membranes varies as a function of 16 17growth rate and in response to changes in the environment. While growth adaptation can be explained by biochemical feedbacks in the PL synthesis pathway, recent 18 transcriptome studies have revealed that the expression of PL synthesis genes can also 19 be tuned in response to various stresses. We previously showed that the BasRS two-20 21component pathway controls expression of the diacylglycerol kinase gene dgkA in Escherichia coli (Wahl et al., Mol. Microbiol., 80:1260-75, 2011). In the present study, we 2223set up a strategy to identify the mutation responsible for the upregulation of *pssA* observed in the historical pssR1 mutant, and supposedly corresponding to a 24transcriptional repressor (Sparrow and Raetz, J. Biol. Chem., 258:9963-7, 1983). pssA is 2526the gene coding for phosphatidylserine synthase, the first step of 27phosphatidylethanolamine synthesis. We showed that this mutation corresponded to a single nucleotide change in the anti- Shine-Dalgarno sequence of the 16S ribosomal 28RNA encoded by the rrnC operon. We further demonstrated that this mutation 2930 enhanced the translation of *pssA*. Though this effect appeared to be restricted to PssA among phospholipid synthesis enzymes, it was not specific, as evidenced by a global 31effect on the production of unrelated proteins. 32

33

34 Importance

Bacteria adjust the phospholipid composition of their membranes to the changing environment. In addition to enzymatic regulation, stress responses regulators control specific steps of the phospholipid synthesis pathway. We wanted to identify a potential regulator controlling the expression of phosphatidylserine synthase gene. We showed that it was not the previously suggested hdfR gene and instead showed that a mutation in the anti-SD sequence of 16S RNA was responsible for an increase in *pssA* translation. This example underlines the fact that gene expression can be modulated by other means than specific regulatory processes.

43 INTRODUCTION

Phospholipids, which are the building blocks of bacterial membranes, are synthesized by 44 45a series of enzymes localized in the inner membrane. The biochemistry of the phospholipid synthesis pathway has been well deciphered, especially in the E. coli model 46 bacterium (1). Phospholipid composition is very tightly controlled, and is modulated by 47various parameters such as growth conditions, growth rate or stresses. Most of this 48control is thought to occur at the enzymatic activity level (1). However, transcriptomic 49 data have recently uncovered the fact that several signaling pathways might control 5051phospholipid synthesis through a regulation of enzyme expression levels in E. coli. 52Indeed, the expression of all the genes for phospholipid synthesis seems to correlate with the growth rate through ppGpp and stringent response control (2, 3). Furthermore, 5354different stress response pathways control specific steps of phospholipid synthesis. For example, the alternative sigma factor σ^{s} activates the expression of *cfa* coding for 5556cyclopropane synthase (4, 5). Membranes are indeed enriched in cyclopropanated phospholipids in stationary phase. Another example is the activation of the expression of 57*plsB* by σ^{E} during envelope stress (6, 7). 58

Interestingly, some time ago, Raetz and collaborators performed extensive mutagenesis screens to isolate genes involved in phospholipid synthesis (8). Among them, they were able to isolate potential regulators of specific steps of the pathway. Notably, one mutant showed an increased expression of dgkA (8, 9). We were able to trace down this mutant to a point mutation in the BasS sensor protein, and showed further that dgkA is part of the BasRS regulon (7). Following this first identification, we then decided to focus on a second regulator that was reported to control the expression 66 of pssA (10). In this study, a mutant exhibiting a specific increased PssA expression was isolated. This mutation was clearly functioning in trans and the authors made the 67 hypothesis of a mutation in a putative PssR repressor of *pssA* expression. The position of 68the mutation was precisely mapped by three-point analysis of random general 69 transduction, at 84 minutes on the chromosome, just upstream the *ilv* gene locus (10). 70Yet, the *pssR* gene was never cloned or identified afterwards. Following genome 7172sequencing and annotation, because of this initial physical mapping of pssR, an ORF was 73annotated as pssR at a certain time. This first annotation was later the object of 74corrections and the *vifA* and *pssR* neighbor ORFs were fused into one single hdfR75ORF(11, 12). However, no effect of *hdfR* mutant on phospholipids was observed (11).

The goal of our study was then to identify pssR and elucidate the regulation of pssA expression. Instead of finding a specific transcriptional regulator, we showed that a single mutation in the anti-Shine-Dalgarno (SD) sequence of 16S rRNA coded by the rrnC operon is responsible for the high amount of PssA enzyme observed in the original mutant strain. This effect, while very strong on PssA, is not specific since the expression of several unrelated genes is also affected.

82 **RESULTS**

83 Increase of *pssA* expression in the AC5 *pssR1* mutant strain

Using the AC1 (pssR+) and AC5 (pssR1) strains provided by the Raetz's laboratory (10), 84we first wanted to reproduce the upregulation of *pssA* reported in the original paper. To 85this end, we used translational fusion of the whole promoter and open reading frame 86 87 (ORF) of *pssA* gene with GFP, on a plasmid. We observed a 6-fold overexpression of 88 pssA in the AC5 strain compared to AC1 (Figure 1A). Then, we also wanted to verify that the same effect was obtained for the amounts of the PssA protein expressed from its 89 90 natural locus on the chromosome. We constructed recombinant strains in which the endogenous PssA was tagged at its C-terminus with the Sequential Peptide Affinity 91 92(SPA) tag, while still expressed from its natural promoter. The amount of PssA-SPA protein as revealed by Western blot was increased 4-fold in the AC5/pssR1 genetic 93 94background compared to the parental AC1 background (Figure 1B, 1C).

95To test if the *pssR1* mutation was indeed linked to the *ilv* genetic locus identified in the original study, and to assess if the upregulation effect was independent of the 96 specific genetic background of the strains, we transduced the *pssR1* mutation from the 97 AC5 strain to the wild type MG1655 reference strain. For this, we first introduced a 98 99 chloramphenicol resistance cassette to replace the hdfR gene ORF through direct 100 recombination, both in the AC1 and AC5 strains. The hdfR gene is located close to the 101 ilv locus, at about 10'000 base pairs. The cassette insertion did not interfere with the 102 upregulation of *pssA* in the AC5 genetic background (Figure 1A, compare EB356/EB886 with AC1/AC5). The $\Delta h df R$:: cat marker was then transduced from the 103 two strains into MG1655. pssA was found to be upregulated in the EB430 strain 104

obtained from the AC5 transduction compared to the EB1011 strain obtained from the AC1 transduction (Figure 1A). This confirmed that pssR1 was genetically linked to the hdfR gene, but suggested that pssR1 was distinct from hdfR (see below). Furthermore, this showed that the effect of the mutation on pssA expression was independent of the particular genetic background of the strain used.

Sparrow and Raetz originally suggested that the observed regulation was 110 specific to *pssA* because the enzymatic activities of 6 other PL synthesis enzymes were 111 not modified (the activities tested at that time corresponded to PlsB, CdsA, PgsA, PGP, 112 113 DgkA, and Psd enzymes) (10). In order to test directly that the production of other PL 114 synthesis enzymes than PssA was not affected, we followed the same strategy used to 115 follow PssA protein amounts, building recombinant strains expressing PlsB-SPA, 116 PlsC-SPA, or PgsA-TAP chimeric proteins (13). Contrary to PssA, and in accordance to 117 the observations of Sparrow and Raetz, the amounts of these three proteins were not modified by the presence of the pssR1 mutation (Figure 1B, 1C). 118

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120 Identification of the mutated gene causing *pssA* over-expression

To identify the location of the *pssR1* mutation, we performed genome resequencing of AC1 and AC5 strains. Compared to the MG1655 reference genome, about 200 point mutations were found throughout the AC1 and AC5 genomes, yet all these mutations were identical in AC1 and AC5. In contrast, 19 mutations were found only in AC5, all restricted to a region between positions 3,923,000 and 3,996,000 of the MG1655 reference genome (Table 1). This region is compatible with the window of about 100 kb corresponding to the P1 transduction used to isolate the *pssR* locus (10). This also highlights the high mutagenesis rate that was used in the original paper. Our whole genome sequencing results thus appeared perfectly consistent with the conclusions obtained from the initial genetic experiments.

The next step was then to identify among the 19 mutations, the one(s) 131 responsible for pssA upregulation. The position of the G237S mutation found in hdfR132ORF in AC5 (Table 1) was in very good agreement with the genetic mapping of pssR (i.e. 133 84 minutes, close to the *ilv* genes). At some point, hdfR was even annotated as potential 134 pssR (12). This, besides the fact that it encodes a transcriptional regulator of the LysR 135 136 family, made HdfR a very good candidate for the PssR repressor that we were looking 137 for. However, when we measured the expression of *pssA* in a $\Delta hdfR$ strain compared to wild type strain, no effect was observed (Figure 2A, compare the pBAD bars). This was 138 139 expected, as the deletion of hdfR did not suppress the difference of *pssA* expression in 140 AC1 and AC5 strains (Figure 1A). To test further if the G237S mutation in HdfR had a specific effect, we cloned the wild type and mutated versions of hdfR in the pBAD24 141 142expression vector. Wild type and mutated HdfR proteins were produced in similar 143 amounts upon induction with arabinose (Figure 2C). We did not observe any significant effect on pssA expression, neither with wild type HdfR nor with HdfR(G237S) mutant 144 overproduction (Figure 2A). To test if the G237S mutation in HdfR had any effect on 145HdfR function, we first tried to use a transcriptional fusion of gltB, a gene whose 146 expression was reported to be regulated by HdfR (14), but we did not observe any 147signal for this fusion in any of the conditions tested (data not shown). Transcriptional 148149 regulators of the LysR family often regulate a divergently transcribed neighboring gene (15). Therefore, we tested the expression of the yifE gene, localized next to hdfR in 150opposite orientation. YifE has been recently renamed MaoP, as it was shown to be 151

152involved in macrodomain organization of the Ori region (16). Interestingly, hdfR and *yifE* genes have been both identified in a genetic screen for genes promoting the stress-153 induced mutagenesis response in E. coli (17). We observed that HdfR strongly activates 154*yifE* expression (Figure 2B). Indeed, *yifE* expression was abolished in a $\Delta h df R$ mutant, 155while it was upregulated if HdfR is overproduced (Figure 2B). However, it was clear 156 that the G237S mutation of HdfR had no effect on *yifE* expression (Figure 2B). In 157 summary, the G237S mutation in HdfR does not seem to influence HdfR function. 158Furthermore overproduction or deletion of hdfR had no effect on *pssA* expression, 159demonstrating that hdfR is not *pssR*. 160

161 Using a similar approach to the one detailed above for HdfR (overproduction of wild type or mutant versions of the proteins, or deletion of the genes), we also ruled out 162163 any link of rsmG(G13S) or rbsA(T173I) mutations (Table 1) with *pssR* (data not shown). Finally, we used an MG1655 strain containing a $\Delta yifK$: kana resistance cassette localized 164 between wzyE and aslA genes at about 30 kb from hdfR (Table 1), in order to transduce 165 166 back into AC5 wild type regions of the chromosome together with the $\Delta yifK$:kana^R 167 marker. The transductants were then screened by measuring the expression of *pssA* and by sequencing the genes originally mutated in AC5. Using this strategy, we were able 168 to obtain a strain (EB1068) with all the genes from hdfR to rrlA that were mutated in 169 AC5 (Table 1) restored to a wild type sequence. This strain still displayed an 170 overproduction of PssA (data not shown). Therefore, we finally investigated the 171 possibility that the only mutations left for study, the ones in the *rrnC* ribosomal operon 172173localized just upstream hdfR on the chromosome (Table 1, Figure 3A), might be responsible for the overproduction of PssA. We deleted the whole *rrnC* operon, or only 174175 the rrsC or rrlC genes by direct recombination in the AC1 and AC5 strains. When rrlC (coding for 23S RNA) was deleted alone, leaving the mutation in *rrsC*, the overproduction of PssA was still observed in the AC5 background compared to the AC1 background (Figure 3B). However, when the whole operon or only the *rrsC* gene was deleted, no overproduction of PssA was observed anymore in the AC5 background (Figure 3B). This left the single mutation C1538T at the extremity of *rrsC* as the only one that could be responsible for PssA overproduction. This mutation is located at the 3' extremity of 16S RNA, in the anti-SD sequence (Figure 3A).

To finally prove that the mutation C1538T in *rrsC* was by itself responsible for 183 the upregulation of *pssA*, we transformed AC1 and AC5 strains by plasmids bearing the 184 185 whole rrnC operon, with or without the C1538T mutation in rrsC. The wild type rrnCplasmid strongly reduced the upregulation of *pssA* in AC5 (Figure 3C), whereas the 186 187 mutated rrnC plasmid provoked the upregulation of pssA in AC1 (Figure 3C). It was 188 impossible to obtain clones from the transformation of AC5 with the mutated rrnC plasmid, suggesting that this mutation C1538T in the anti-SD sequence had deleterious 189 effects, as already reported (18). 190

191 The C1538T mutation in AC5 increases *pssA* translation level

Given the nature of the mutation responsible for the overexpression of *pssA*, we suspected that the regulation happened at the initiation of translation. To test this, we dissected the promoter and 5'UTR regions of *pssA* to produce different transcriptional or translational fusions with GFP (Figure 4A). The transcriptional start site of *pssA* has been mapped 59 nucleotides before its ATG start codon in a global RNAseq study (19). In order to verify this position, we first mutated the corresponding -10 promoter sequence in the *pssA*-GFP translational fusion (F1, Figure 4A). The mutation abolished

199 the expression of psA (Figure 4B), confirming the +1 position, which we then used as 200 the limit for constructing strict transcriptional or translational fusions (respectively F2 and F3, Figure 4A). The expression of GFP under the control of the promoter of *pssA* 201 202 and an artificial mRNA leader sequence was not very different in AC1 or AC5 strains (Figure 4C, PpssA i.e. F2 fusion). On the contrary, the expression of GFP under the 203 control of the Plac promoter and the 5'UTR of pssA was increased 6-fold in AC5 204 205 compared to AC1 (Figure 4C, 5'UTR i.e. F3 fusion), which is very similar to the 4 to 5 fold increase observed with the global *pssA*-GFP fusion (Figure 4B and 4C, F1 fusion). 206 This demonstrated that the effect of the pssR1 mutant on pssA expression was post-207208 transcriptional. We reasoned that the C1538U mutation in the anti-SD 16S RNA sequence might allow a better recognition of the SD sequence of *pssA*. Indeed, the SD 209 sequence of psA is not optimal, but the C1538U mutation improves the base pairing at 2 210 putative positions on *pssA* : AGAAG or AAGAA (Figure 4A). We tried to restore a 211212 better base pairing with the wild type 16S RNA by introducing a mutation at the second 213position in the *pssA* SD sequence (Figure 4A). The mutation abolished over-expression of *pssA* in AC5 (Figure 4B). However, though designed as a compensatory mutation, it 214 215 did not trigger in AC1 an expression as strong as that of wild type *pssA* in AC5. Another 216 possibility is that this mutation may simply increase by 1 nuceotide the base pairing of 217the upstream position.

Together, these results demonstrated that the initiation of *pssA* translation is increased in AC5, presumably due to an enhanced base pairing of the SD sequence of *pssA* with the C1538T mutated anti-SD sequence of 16S RNA.

221 Differential proteome analysis of the AC1 and AC5 strains

Since we showed that the psR1 genotype could be reduced to the C1538T mutation in 222223rrsC, it was difficult to imagine that the effect was restricted to *pssA* expression. Indeed, the C1538T mutation map at the extremity of 16S RNA, in the anti-SD sequence, and 224was thus expected to affect the translation of a larger number of genes. It has to be 225noted that this mutation was studied before, shown to be deleterious, and also described 226 to affect the expression of several genes (18). We wanted to obtain a global view of the 227genes whose expression was impacted by the mutation. To this end, we performed a 228229 quantitative proteomic analysis of the AC1 and AC5 strains to identify differentially 230 expressed proteins. This permitted to identify and quantify 1318 proteins (Table S1). Stringent statistical analysis revealed 85 proteins whose expression levels were modified 231232at least 2 fold between the compared strains (highlighted in green and blue in Table S1). 233Thus, 43 proteins were found more expressed in AC5 strain, whereas 42 were found enriched in AC1 strain. Among the differentially expressed proteins, PssA was found 234over-expressed more than 6 times (Log_2 (Fold Change) = 2.75) in AC5 compared to 235236 AC1, which technically validated the experiment.

In order to validate these data, we choose in this table several proteins (highlighted in bold in Table S1) for which a strain was available in a collection of *E. coli* -SPA tagged strains (13). The *holC*, *aspA*, *smpB*, *ybbA*, and *ygeV*-SPA tagged constructions were transduced in the AC1 and AC5 strains and the amounts of the proteins were quantified by Western blot using a monoclonal antibody directed against the SPA tag. We confirmed the increase of HolC, YgeV, YbbA, and AspA protein expression in AC5 strain compared to AC1 strain while, in contrast with the observation of the proteomics analysis, SmpB-SPA levels were found to decrease in AC5
strain (Figure S1).

Therefore, while PssA was apparently the only PL synthesis enzyme upregulated in the pssR1 mutant ((10) and our results), the mutation in the anti-SD sequence of 16S RNA encoded by the rrnC operon affected the translation of several other genes.

Global effect of the C1538T mutation in 16S RNA on gene expression and

252 physiology

The C1538T mutation has been reported to be strongly toxic, as the mutated rRNA 253operon could not be expressed from a plasmid even in a wild type strain (18). In contrast, 254255we were able to transform the wild type AC1 by the mutated plasmid (using a plasmid 256similar to the one used in (18)) (Figure 3C). We did observe a slight growth phenotype 257for the AC5 strain in which the mutated rmC operon is expressed from its natural 258chromosomal locus. And it was indeed impossible to transform further the AC5 strain by a plasmid bearing the same mutated rRNA operon, demonstrating the deleterious 259effect of the mutation. In the same study, a series of proteins whose production was 260affected by the mutation were identified by comparative proteomic 2D gel analysis (18). 261From the 8 proteins reported to be affected by the mutation, we only found Methionyl-262tRNA synthetase as significantly enriched in our proteomic analyses of the AC1 and 263264 AC5 strains (MetG, Table S1). This discrepancy might be explained by the different levels of expression of the mutated rrnC operon (inducible plasmid in (18), expression 265from the chromosomal locus in our study), but also by the low accuracy of the method 266used previously. We are confident in our proteomics analysis, given that PssA was 267 identified, and that 4 proteins randomly chosen in the set confirmed to be similarly 268affected in a different experiment. We only obtained a contradictory result for SmpB 269 270 protein. However, given the role of SmpB in trans-translation (20) a possible 271explanation is that the -SPA tag might have perturbed the function of SmpB protein, which in return would affect broadly the translation of many proteins including itself. 272

273In (18), the authors hypothesized that the amount of protein produced reflected 274the change in complementarity between the mutated rRNA and the SD sequence in the mRNA. When we looked at the proteins we identified by proteomics analysis, apart 275276from PssA (see below), it was difficult to pinpoint specific features of the SD sequences for the overproduced or reduced proteins. In some cases it was possible to correlate 277upregulation or donwregulation with a better or worse base pairing with the mutated 27816S RNA, as described in (18). However, the expression of many genes might be 279indirectly affected by the increased or decreased translation of others. Furthermore, 280because we performed the analysis in AC1 and AC5 strains, it is possible that the 281282additional mutations present in AC5 (Table 1) might also affect the expression of some 283genes.

PssA translation is affected by the C1538T mutation in 16S RNA

In this study, we showed that a mutation in the anti-SD sequence of the 16S RNA 285encoded by the rrnC operon results in a strong increase of PssA translation. We 286287demonstrated that this mutation corresponds to the putative pssR regulator described in (10). These results completely rule out the hdfR gene as a candidate for pssR and 288indicate that HdfR is not involved in lipid metabolism regulation. HdfR has been 289reported to be a negative regulator of the flagellar master regulator *flhDC* and activates 290 the gltBD operon (11, 14). It might be involved in a complex regulatory network 291involving H-NS and other regulators involved in acid stress resistance (14). However, 292293its binding site has not been identified and we could not observe a regulation of the 294gltBD promoter. In contrast, it seems that the adjacent *yifE* gene might be a genuine HdfR target gene, given its strong regulation and its classical genetic organization 295relative to hdfR. It has been shown recently that YifE protein (renamed MaoP) is 296

involved in the chromosome organization of the Ori macrodomain by binding to the specific maoS sequence localized in the hdfR-yifE intergenic region (16). It will be very interesting to understand the role of HdfR regulation and its connection with the MaoP/maoS site-specific control of the Ori macrodomain.

The fact that mutation C1538T in the 16S RNA rrsC coding gene corresponds to 301 the pssR1 allele meant that we did not find a specific regulator of pssA expression. While 302 303 no other phospholipid synthesis gene than *pssA* seemed to be affected by the mutation, its nature excludes a specific effect. Indeed, we showed by proteomics analyses that the 304 305 expression of several genes was affected. However, we did not systematically verified if it was a direct effect on their translation. We suggest that the effect of the C1538T 306 mutation depends on the nature of the SD sequence of a given gene. The strong effect 307 308 on pssA translation reflects the suboptimal nature of the SD sequence of pssA, which 309 might be better recognized by the mutated anti-SD sequence of 16S RNA. This weak 310 initiation of translation might be important in the control of *pssA* expression and highlights the fact that gene expression can be modulated by other means than specific 311 312 regulatory processes.

313 MATERIALS AND METHODS

314 Strain Constructions

For all strain and plasmid constructions, the Ecocyc website (21) was used for sequence 315 retrieval. The construction of the various strains is described succinctly in Table 2. 316 Deletions of hdfR, rrnC, rrlC, or rrsC were performed by direct recombination of PCR 317 318 fragments obtained using pKD3 or pKD4 plasmids as templates, following the Datsenko 319 and Wanner procedure (22). Deletion mutant alleles obtained by recombination or from the Keio collection (23), or from tagged strains from the DY330 strain collection (13), 320 321were transduced from one background to another by generalized transduction with the phage P1 (24). 322

323 Plasmid Constructions

Plasmid constructions are described succinctly in Table 3. The hdfR ORF was amplified by PCR using AC1 and AC5 genomic DNA as templates and cloned in the pBAD24 vector to obtain plasmids pEB1408 and pEB1501 respectively. Plasmid pcSacB-*rrnC* that contains the entire *rrnC* operon was PCR mutagenized to obtain pcSacB-*rrnC*(C1538T).

Transcriptional or translational fusions with GFP were constructed in the pUA66 vector backbone (25). When available, transcriptional fusions were retrieved from the Zaslaver collection (25), or else the promoter regions were PCR amplified and cloned between XhoI and BamHI restriction sites. The pssA-GFPmut2 translational fusions were also transferred in the pZE plasmid (26).

334 Measure of expression using transcriptional and translational fusions with GFP

E. coli strains were transformed by the indicated plasmids derived from pUA66 (25) or 335336 pZE-GFPmut2 (pEB1900) plasmids, and the selection plates were incubated at 37°C for 16 h. Six hundred microliters of LB medium supplemented with the required antibiotics 337 were inoculated (4 to 6 replicates each assay) and grown for 16 h at 30°C in 96-well 338 polypropylene plates of 2.2-ml wells under aeration and agitation. Fluorescent intensity 339 measurement was performed in a Tecan infinite M200 plate reader. One hundred fifty 340 microliters of each well was transferred into a black Greiner 96-well plate for reading 341 342optical density at 600 nm (OD600) and fluorescence (excitation, 485 nm; emission, 530 343 nm). The expression levels were calculated by dividing the intensity of fluorescence by 344the OD600. After mean values were calculated, values from the control vector were 345subtracted. The results are given in arbitrary units, because the intensity of fluorescence 346 is acquired with an optimal and variable gain; hence, the absolute values cannot be compared between different types of experiment and growth conditions. 347

348 AC1 and AC5 strain genome resequencing

Genomic DNA of AC1 and AC5 strains was prepared using the Wizard Genomic DNA Purification Kit from Promega. Library construction, Illumina sequencing, and data analysis were performed by GATC Biotech.

352 Proteomics analysis of AC1 and AC5 strains

AC1 and AC5 strains were grown in duplicate in 100ml LB at 37°C until OD600 = 2. Protein preparation and mass spectrometry-based proteomic analyses were realized as described in (27). Briefly, extracted proteins were stacked in the top of a SDS-PAGE gel (NuPAGE 4-12%, Invitrogen) before in-gel digestion using trypsin (Promega,

357 sequencing grade). Resulting peptides were analyzed in duplicates by online nanoLC-MS/MS (UltiMate 3000 and LTO-Orbitrap Velos Pro, Thermo Scientific) using a 120-358min gradient. Peptides and proteins were identified and quantified using MaxQuant 359 (version 1.5.7.4, (28)) and Uniprot database (February 2017 version, Escherichia coli K12 360 taxonomy). For statistical analysis, we used ProStaR (29): proteins identified in the 361 reverse and contaminant databases and proteins exhibiting less than 4 intensity values 362in one condition were discarded from the list. After log2 transformation, intensity 363 values were normalized by median centering before missing value imputation (replacing 364 missing values by the 1 percentile value of each column); statistical testing was 365366 conducted using *limma* t-test. Differentially expressed proteins were sorted out using a log2(fold change) cut-off of 1 and a FDR threshold on p-values of 1% using the 367 Benjamini-Hochberg method. 368

369 SDS-PAGE and Western blotting

SDS-PAGE, electrotransfer onto nitrocellulose membranes, and Western blot analyses were performed as previously described (30). Monoclonal anti-Flag M2, used for SPA-tag detection, and PAP antibody used for TAP-tag detection, were purchased from Sigma. The relative amounts of FA synthesis enzymes fused to the SPA tag or TAP tag were quantified by 10% SDS-PAGE and Western blotting. The amounts produced were then quantified using Alexa Fluor 680 anti-mouse IgG fluorescent secondary antibodies (Invitrogen) on an Odyssey Fc imager from LI-COR Biosciences.

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 492 Bacteriol 177:4121-4130.

494 Table 1 : Genome sequence differences between AC1 and AC5 strains identified

- 495 by genome resequencing
- 496 a : Positions of the mutations on the *E. coli* K-12 MG1655 reference genome as given in
- 497 Ecocyc database (21).
- b : Missense mutations and the corresponding genes are highlighted in bold. *yifK* gene
- 499 used for mapping pssR1 is also indicated.

| Position ^a | AC1 | AC5 | AA change ^b | Gene ^b |
|-----------------------|-----|-----|------------------------|-------------------|
| 3923644 | С | Т | G13S | rsmG |
| 3926991 | G | А | L5 | asnC |
| 3934295 | С | Т | T173I | rbsA |
| 3935773 | С | Т | G162 | rbsC |
| 3943345 | С | Т | NA | rrsC |
| 3945437 | С | Т | NA | rrlC |
| 3945582 | С | Т | NA | rrlC |
| 3946315 | С | Т | NA | rrlC |
| 3947259 | С | Т | G237S | hdfR |
| 3979126 | С | Т | R176C | wzyE |
| | | | | yifK |
| 3982939 | С | Т | NA | intergenic |
| 3985435 | С | Т | M191I | aslA |
| 3988482 | С | Т | S195 | hemX |
| 3990406 | С | Т | A121T | hemC |
| 3993390 | С | Т | F746 | cyaA |
| 3993903 | А | G | C53R° | cyaY |
| 3994950 | С | Т | F63 | dapF |
| 4035734 | Т | G | NA | rrsA |
| 4038695 | Т | С | NA | rrlA |

500 c: In the MG1655 reference strain, residue 53 is an Arginine

501

| Lab code | Name | Description | Reference |
|---------------|---------------------------------------|--|------------|
| EB944 | MG1655 | Wild type parental <i>E. coli</i> K-12 strain | |
| EB801 | BW25113 $\Delta h df R$::kanaR | | (23) |
| EB466 | BW25113 ΔyifK::kanaR | | (23) |
| EB814 | MG1655 $\Delta h df R^{\circ}$ | Transduction $\Delta h df R$:: kanaR from EB801 to | This work |
| | | MG1655; cassette removed using pCP20 | |
| | | plasmid | |
| EB815 | AC1 | pssR+ | (10) |
| EB816 | AC5 | pssR1 | (10) |
| EB356 | AC1 $\Delta hfdR::cat$ | PCR ebm1094/1081 on pKD3 of the <i>cat</i> cassette | This work |
| | , , , , , , , , , , , , , , , , , , , | recombined in AC1 | |
| EB886 | AC5 $\Delta h df R$::cat | PCR ebm1094/1081 on pKD3 of the <i>cat</i> cassette | This work |
| | U U | recombined in AC5 | |
| EB1011 | $MG\Delta hdfR::cat pssR+$ | Transduction $\Delta h df R$:: cat from EB356 to | This work |
| | | MG1655 | |
| EB430 | $MG\Delta hdfR::cat pssR1$ | Transduction $\Delta h df R$:: cat from EB886 to | This work |
| | | MG1655 | |
| EB1068 | AC5∆yifK::kanaR (JB70) | <i>pssR1, hdfR</i> wt. Transduction ∆ <i>yifK</i> ::kanaR from | This work |
| | | EB466 to EB886 | |
| | DY330 series | Collection of strains with -SPA or -TAP tags | (13) |
| | | on the chromosome | |
| EB160 | AC1/PssA-SPA | Transduction of <i>pssA</i> -SPA-kanaR from the – | This work |
| | | SPA collection to AC1 | |
| EB161 | AC5/PssA-SPA | Transduction of <i>pssA</i> -SPA-kanaR from the – | This work |
| | | SPA collection to AC5 | |
| EB1052 | AC1/PlsB-SPA | Transduction of <i>plsB</i> -SPA-kanaR from the – | This work |
| | | SPA collection to AC1 | |
| EB1053 | AC5/PlsB-SPA | Transduction of <i>plsB</i> -SPA-kanaR from the – | This work |
| | | SPA collection to AC5 | |
| EB1050 | AC1/PlsC-SPA | Transduction of <i>plsC</i> -SPA-kanaR from the – | This work |
| | | SPA collection to AC1 | |
| EB1051 | AC5/PlsC-SPA | Transduction of <i>plsC</i> -SPA-kanaR from the – | This work |
| | | SPA collection to AC5 | |
| EB1054 | AC1/PgsA-TAP | Transduction of $pgsA$ -TAP-kanaR from the – | This work |
| | | SPA collection to AC1 | |
| EB1055 | AC5/PgsA-TAP | Transduction of <i>pgsA</i> -TAP-kanaR from the – | This work |
| | | SPA collection to AC5 | |
| EB350 | AC1ΔrrnC | PCR ebm1624/1620 on pKD4 recombined in | This work |
| DD | | AC1 | |
| EB352 | AC5∆rrnC | PCR ebm1624/1620 on pKD4 recombined in | This work |
| FD ata | A G: A 10 | | |
| EB349 | ACIΔrriC | PCR ebm1619/1620 on pKD4 recombined in | I his work |
| EDari | A.G.Z.A. 10 | ACI | |
| EB351 | AC5ΔrrlC | PCR ebm1619/1620 on pKD4 recombined in | I his work |
| EB1002 | A C 1 A mur C | ACD PCD short 2004 / 1040 ser VD4 12 12 | This and |
| EB1062 | AUIArrsU | PUR ebm1624/1640 on pKD4 recombined in | I his work |
| EP10ce | ACEAnnoC | $\frac{DCP}{DCP} = \frac{1}{2} \frac{1}{1} \frac{1}{1$ | This work |
| ED1063 | AUDAITSU | FUR eDm1624/1640 on pRD4 recombined in | 1 nis work |
| | | AU3 | 1 |

503 Table 2: E. coli K-12 strains

505 Table 3: Plasmids

- 506 a: Trad signifies translational fusion. b: Ts : thermosensitive replication; ori : origin of
- 507 replication; Δrbs : eletion of ribosome binding site on the vector

| Lab code | name ^a | description ^b | Reference |
|----------|--------------------------------|---|-------------------------|
| pEB0268 | pKD3 | ampiR, FRT-camR-FRT cassette | (22) |
| pEB0269 | pKD4 | ampiR, FRT-kanaR-FRT cassette | (22) |
| pEB0267 | pKD46 | Ts, ampiR, lambda Red genes | (22) |
| pEB0266 | pCP20 | Ts, ampiR, camR, FLP recombinase gene | (31) |
| pEB0227 | pBAD24 | ampiR, PBAD promoter | (32) |
| pEB1408 | pBAD-hdfR | PCR with ebm870/871 oligonucleotides on AC1 genomic DNA (EcoRI/XhoI) in pBAD24 (EcoRI/SalI) | This work |
| pEB1501 | pBAD-hdfR(G237S) | PCR with ebm870/871 oligonucleotides on AC5 genomic DNA (EcoRI/XhoI) in pBAD24 (EcoRI/SalI) | This work |
| pEB0898 | pUA66 | kanaR, sc101 ori, GFPmut2 | (25) |
| | pUA-UP <i>yifE</i> | Region -196/+99 relative to the start codon of yifE | (25) |
| pEB1427 | pTrad-GFPmut2 | <i>kanaR</i> , sc101 ori, Δrbs, PCR mutagenesis on pUA66 with ebm927/928 oligonucleotides | This work |
| pEB1611 | pTrad-pssA(F1) | <i>kanaR</i> , sc101 ori, PCR with 427/1192 oligonucleotides in pEB1427 (XhoI/BamHI) | This work |
| pEB1895 | pUA-Plac5'UTR <i>pssA</i> (F3) | kanaR, sc101 ori, PCR with ebm1656/1657 oligonucleotides in pEB1427 | This work |
| pEB1856 | pUA-PpssA (F2) | kanaR, sc101 ori, PCR with ebm1205/1405 oligonucleotides in pUA66 (XhoI/BamHI) | This work |
| pEB1323 | pZEgfp | ampiR, pBR322 ori, GFPmut3aav | (26) |
| pEB1900 | pZE-Trad-GFPmut2 | ampiR, pBR322 ori. Δrbs. pEB1427 (XhoI/XbaI) insert in pEB1323 (XhoI/XbaI) | This work |
| pEB1853 | pPssA-GFPmut2 (F1) | ampiR, pBR322 ori, PCR with 427/1192 oligonucleotides in pEB1900 (XhoI/BamHI) | This work |
| pEB1864 | pPssAmut10 | ampiR, PCR mutagenesis on pEB1853 with ebm1631/1632 oligonucleotides | This work |
| pEB1878 | pPssA_mutSD | ampiR, PCR mutagenesis on pEB1853 with ebm1654/1655 oligonucleotides | This work |
| pEB1858 | pcSacB- <i>rrnC</i> | kanaR | S. Quan, unpublished |
| pEB1866 | pcSacB-rrnC rrsC(C1538T) | PCR mutagenesis on pEB1858 with ebm1641/1642 oligonucleotides | This work |

509 Figure legends

510

511Figure 1 : Upregulation of pssA in the pssR1 mutant. A. Strains AC1 (pssR+), AC5 512(pssR1), AC1 $\Delta hdfR$::camR (EB356); AC5 $\Delta hdfR$::camR (EB886), MG $\Delta hdfRpssR$ + (EB1011), and MG $\Delta hdfRpssR1$ (EB430) (Table 2) were transformed with the *pssA*-GFP 513514translational fusion and the corresponding control (plasmids pEB1853 and pEB1900 respectively), and expression of *pssA*-GFP was measured as described in Materials and 515Methods. Data are the mean of four replicates each and the error bars show the standard 516deviations. B. Strains AC1 and AC5 producing tagged phospholipid enzymes, EB160 517and EB161 (PssA-SPA), EB1052 and EB1053 (PlsB-SPA), EB1050 and EB1051 518(PlsC-SPA), and EB1054 and EB1055 (PgsA-TAP) (Table 2), were grown in LB at 519 37°C and stopped in exponential growth phase. Whole cell extracts were separated on 52010% SDS-PAGE and the SPA-tagged or TAP-tagged proteins were analyzed by 521Western blot using anti-Flag or PAP antibodies respectively. C. The experiment shown 522in B. was repeated with three or four replicates for each strain, and the Western blots 523were quantified on a Li-Cor Imager. The error bars show the standard deviations. 524

525

Figure 2 : hdfR is not pssR. A. Wild type MG1655 strain was transformed by the pBAD24, pBAD-hdfR or pBAD-hdfR(G237S) plasmids. Cells were grown in LB at 37°C and expression was induced at 0.5% arabinose during 2 hours. Whole cell extracts were separated on 10% SDS-PAGE, and proteins were stained with Coomassie blue. Strains MG1655 and $\Delta hdfR$ (EB814) were transformed simultaneously with the indicated fusions pTrad-*pssA* (pEB1611) (**B**) or pUA-*yifE* (**C**), and the pBAD-*hdfR* or pBAD-*hdfR*(G237S) plasmids (Table 3). Cultures were performed in LB supplemented with ampicillin, kanamycin, and 0.5% arabinose and expression was monitored as
described in the Materials and Methods. Data are the mean of 4 replicates each and the
error bars show the standard deviations.

Figure 3 : rrsC(C1538T) mutation is responsible for the increase in *pssA* 536expression in AC5. A. rrnC operon. The positions of the mutations present in AC5 537but not in AC1 are indicated in red. B. Strains AC1 or AC5 deleted of the portions of 538the rrnC operon indicated (strains EB350, EB349, EB1062, EB352, EB351, EB1063, see 539Table 2) were transformed with the *pssA*-GFP translational fusion (plasmid pEB1853), 540 541and expression was monitored as described in Materials and Methods. Data are the 542mean of 4 replicates each and the error bars show the standard deviations. C. Strains AC1 or AC5 were transformed with the *pssA*-GFP reporter fusion (plasmid pEB1853) 543544and the indicated plasmids (cont: pEB0354; prrnC: pEB1858; prrnC(C1538T): pEB1866). 545Expression was monitored as described in Materials and Methods. Data are the mean of 546 4 replicates each and the error bars show the standard deviations.

547

Figure 4 : rrsC(C1538T) mutation affects pssA translation initiation. A. 5'UTR 548sequence of *pssA* and wild type or mutated anti-SD sequences of 16S RNA are shown. 549Mutations are indicated in red. TSS : transcription start site. The different 550551constructions of GFP fusions with portions of *pssA* gene are shown below. Regions 552coming from the *pssA* gene are drawn in black; Plac promoter is drawn in blue, and 553artificial ribosome binding site (rbs) and GFP sequences are drawn in green. B and C. Strains AC1 or AC5 were transformed with the indicated reporter fusions (plasmids 554pEB1853, pEB1864, and pEB1878 (B); plasmids pEB1611, pEB1856, and pEB1895 (C)). 555

- 556 Expression was monitored as described in Materials and Methods. Data are the mean of
- 557 4 replicates each and the error bars show the standard deviations.

Figure 1



Figure 2

Α hdfR(G237S) ni l pBADhdfR 'ni ni L I -HdfR 25kDa В pTrad-pssA Fluorescence / OD_{600nm} (A.U.) MG1655 Δ hdfR Port Draft Costs Porus un Astrony PBAD С pUA-yifE Fluorescence / OD_{600nm} (A.U.) 100000 MG1655 80000 ____∆hdfR 60000 40000 PERO HURRESSE Port Draft Constant 20000

Figure 3



Α

