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

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The Long Hunt For \textit{pssR} – looking for a phospholipid synthesis transcriptional regulator, finding the ribosome.

Running title: \textit{pssR}1 phenotype due to an anti-SD sequence mutation

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Abstract

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

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.
INTRODUCTION

Phospholipids, which are the building blocks of bacterial membranes, are synthesized by a 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 bacterium (1). Phospholipid composition is very tightly controlled, and is modulated by various parameters such as growth conditions, growth rate or stresses. Most of this control is thought to occur at the enzymatic activity level (1). However, transcriptomic data have recently uncovered the fact that several signaling pathways might control phospholipid synthesis through a regulation of enzyme expression levels in E. coli. Indeed, 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, different stress response pathways control specific steps of phospholipid synthesis. For example, the alternative sigma factor σS activates the expression of cfa coding for cyclopropane synthase (4, 5). Membranes are indeed enriched in cyclopropanated phospholipids in stationary phase. Another example is the activation of the expression of plsB by σE during envelope stress (6, 7).

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
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 hypothesis of a mutation in a putative PssR repressor of *pssA* expression. The position of the mutation was precisely mapped by three-point analysis of random general transduction, at 84 minutes on the chromosome, just upstream the *ile* gene locus (10). Yet, the *pssR* gene was never cloned or identified afterwards. Following genome sequencing and annotation, because of this initial physical mapping of *pssR*, an ORF was annotated as *pssR* at a certain time. This first annotation was later the object of corrections and the *yifA* and *pssR* neighbor ORFs were fused into one single *hdfR* ORF (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.
RESULTS

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), we first wanted to reproduce the upregulation of \( pssA \) reported in the original paper. To this end, we used translational fusion of the whole promoter and open reading frame (ORF) of \( pssA \) gene with GFP, on a plasmid. We observed a 6-fold overexpression of \( pssA \) 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 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 (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 background compared to the parental AC1 background (Figure 1B, 1C).

To 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 specific genetic background of the strains, we transduced the \( pssR1 \) mutation from the AC5 strain to the wild type MG1655 reference strain. For this, we first introduced a chloramphenicol resistance cassette to replace the \( hdfR \) gene ORF through direct recombination, both in the AC1 and AC5 strains. The \( hdfR \) gene is located close to the \( ilv \) locus, at about 10’000 base pairs. The cassette insertion did not interfere with the upregulation of \( pssA \) in the AC5 genetic background (Figure 1A, compare EB356/EB886 with AC1/AC5). The \( \Delta hdfR::cat \) marker was then transduced from the two strains into MG1655. \( pssA \) was found to be upregulated in the EB430 strain.
obtained from the AC5 transduction compared to the EB1011 strain obtained from the
AC1 transduction (Figure 1A). This confirmed that \textit{pssR1} was genetically linked to the
\textit{hdfR} gene, but suggested that \textit{pssR1} was distinct from \textit{hdfR} (see below). Furthermore,
this showed that the effect of the mutation on \textit{pssA} expression was independent of the
particular genetic background of the strain used.

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

\textbf{Identification of the mutated gene causing \textit{pssA} over-expression}

To identify the location of the \textit{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 \textit{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) responsible for pssA upregulation. The position of the G237S mutation found in hdfR ORF in AC5 (Table 1) was in very good agreement with the genetic mapping of pssR (i.e. 84 minutes, close to the ilv genes). At some point, hdfR was even annotated as potential pssR (12). This, besides the fact that it encodes a transcriptional regulator of the LysR family, made HdfR a very good candidate for the PssR repressor that we were looking for. However, when we measured the expression of pssA in a ΔhdfR strain compared to wild type strain, no effect was observed (Figure 2A, compare the pBAD bars). This was expected, as the deletion of hdfR did not suppress the difference of pssA expression in 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 pBAD expression vector. Wild type and mutated HdfR proteins were produced in similar 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 overproduction (Figure 2A). To test if the G237S mutation in HdfR had any effect on HdfR function, we first tried to use a transcriptional fusion of gltB, a gene whose expression was reported to be regulated by HdfR (14), but we did not observe any signal for this fusion in any of the conditions tested (data not shown). Transcriptional 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 opposite orientation. YifE has been recently renamed MaoP, as it was shown to be
involved 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-induced mutagenesis response in E. coli (17). We observed that HdfR strongly activates yifE expression (Figure 2B). Indeed, yifE expression was abolished in a ∆hdfR mutant, while it was upregulated if HdfR is overproduced (Figure 2B). However, it was clear that the G237S mutation of HdfR had no effect on yifE expression (Figure 2B). In summary, the G237S mutation in HdfR does not seem to influence HdfR function.

Furthermore overproduction or deletion of hdfR had no effect on pssA expression, demonstrating that hdfR is not pssR.

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 any link of rsmG(G13S) or rhsA(T173I) mutations (Table 1) with pssR (data not shown).

Finally, we used an MG1655 strain containing a ΔyifK::kana resistance cassette localized between wzyE and aslA genes at about 30 kb from hdfR (Table 1), in order to transduce back into AC5 wild type regions of the chromosome together with the ΔyifK::kanaR 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 to obtain a strain (EB1068) with all the genes from hdfR to rrlA that were mutated in AC5 (Table 1) restored to a wild type sequence. This strain still displayed an overproduction of PssA (data not shown). Therefore, we finally investigated the possibility that the only mutations left for study, the ones in the rrnC ribosomal operon localized 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 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 the upregulation of pssA, we transformed AC1 and AC5 strains by plasmids bearing the whole rrnC operon, with or without the C1538T mutation in rrsC. The wild type rrnC plasmid strongly reduced the upregulation of pssA in AC5 (Figure 3C), whereas the mutated rrnC plasmid provoked the upregulation of pssA in AC1 (Figure 3C). It was 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 effects, as already reported (18).

**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
the expression of \textit{pssA} (Figure 4B), confirming the +1 position, which we then used as
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 \textit{pssA}
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
control of the Plac promoter and the 5′UTR of \textit{pssA} was increased 6–fold in AC5
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 \textit{pssA}-GFP fusion (Figure 4B and 4C, F1 fusion).
This demonstrated that the effect of the \textit{pssR1} mutant on \textit{pssA} expression was post-
transcriptional. We reasoned that the C1538U mutation in the anti–SD 16S RNA
sequence might allow a better recognition of the SD sequence of \textit{pssA}. Indeed, the SD
sequence of \textit{pssA} is not optimal, but the C1538U mutation improves the base pairing at 2
putative positions on \textit{pssA} : AGAAG or AAGAA (Figure 4A). We tried to restore a
better base pairing with the wild type 16S RNA by introducing a mutation at the second
position in the \textit{pssA} SD sequence (Figure 4A). The mutation abolished over-expression
of \textit{pssA} in AC5 (Figure 4B). However, though designed as a compensatory mutation, it
did not trigger in AC1 an expression as strong as that of wild type \textit{pssA} in AC5. Another
possibility is that this mutation may simply increase by 1 nucotide the base pairing of
the upstream position.

Together, these results demonstrated that the initiation of \textit{pssA} translation is increased
in AC5, presumably due to an enhanced base pairing of the SD sequence of \textit{pssA} with the
C1538T mutated anti-SD sequence of 16S RNA.
Differential proteome analysis of the AC1 and AC5 strains

Since we showed that the \textit{pssR1} genotype could be reduced to the C1538T mutation in \textit{rrsC}, it was difficult to imagine that the effect was restricted to \textit{pssA} expression. Indeed, the C1538T mutation map at the extremity of 16S RNA, in the anti-SD sequence, and was thus expected to affect the translation of a larger number of genes. It has to be noted that this mutation was studied before, shown to be deleterious, and also described to affect the expression of several genes (18). We wanted to obtain a global view of the genes whose expression was impacted by the mutation. To this end, we performed a quantitative proteomic analysis of the AC1 and AC5 strains to identify differentially expressed proteins. This permitted to identify and quantify 1318 proteins (Table S1).

Stringent statistical analysis revealed 85 proteins whose expression levels were modified at least 2 fold between the compared strains (highlighted in green and blue in Table S1). Thus, 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 over-expressed more than 6 times (Log2(Fold Change) = 2.75) in AC5 compared to 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 \textit{E. coli} –SPA tagged strains (13). The \textit{holC}, \textit{aspA}, \textit{smpB}, \textit{ybbA}, and \textit{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.
DISCUSSION

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

The C1538T mutation has been reported to be strongly toxic, as the mutated rRNA operon could not be expressed from a plasmid even in a wild type strain (18). In contrast, we were able to transform the wild type AC1 by the mutated plasmid (using a plasmid similar to the one used in (18)) (Figure 3C). We did observe a slight growth phenotype for the AC5 strain in which the mutated \textit{rrnC} operon is expressed from its natural chromosomal locus. And it was indeed impossible to transform further the AC5 strain by a plasmid bearing the same mutated rRNA operon, demonstrating the deleterious effect of the mutation. In the same study, a series of proteins whose production was affected by the mutation were identified by comparative proteomic 2D gel analysis (18). From the 8 proteins reported to be affected by the mutation, we only found Methionyl-tRNA synthetase as significantly enriched in our proteomic analyses of the AC1 and AC5 strains (MetG, Table S1). This discrepancy might be explained by the different levels of expression of the mutated \textit{rrnC} operon (inducible plasmid in (18), expression from the chromosomal locus in our study), but also by the low accuracy of the method used previously. We are confident in our proteomics analysis, given that PssA was identified, and that 4 proteins randomly chosen in the set confirmed to be similarly affected in a different experiment. We only obtained a contradictory result for SmpB protein. However, given the role of SmpB in trans-translation (20) a possible explanation 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.
In (18), the authors hypothesized that the amount of protein produced reflected the 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 from 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 upregulation or downregulation with a better or worse base pairing with the mutated 16S RNA, as described in (18). However, the expression of many genes might be indirectly affected by the increased or decreased translation of others. Furthermore, because we performed the analysis in AC1 and AC5 strains, it is possible that the additional mutations present in AC5 (Table 1) might also affect the expression of some genes.

**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 encoded by the *rrnC* operon results in a strong increase of PssA translation. We demonstrated 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 indicate that HdfR is not involved in lipid metabolism regulation. HdfR has been reported to be a negative regulator of the flagellar master regulator *flhDC* and activates the *gltBD* operon (11, 14). It might be involved in a complex regulatory network involving H-NS and other regulators involved in acid stress resistance (14). However, its binding site has not been identified and we could not observe a regulation of the *gltBD* 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 relative to *hdfR*. It has been shown recently that YifE protein (renamed MaoP) is
involved in the chromosome organization of the Ori macrodomain by binding to the specific \textit{maoS} sequence localized in the \textit{hdfR-yifE} intergenic region (16). It will be very interesting to understand the role of HdfR regulation and its connection with the MaoP/\textit{maoS} site-specific control of the Ori macrodomain.

The fact that mutation C1538T in the 16S RNA \textit{rrsC} coding gene corresponds to the \textit{pssR1} allele meant that we did not find a specific regulator of \textit{pssA} expression. While no other phospholipid synthesis gene than \textit{pssA} seemed to be affected by the mutation, its nature excludes a specific effect. Indeed, we showed by proteomics analyses that the 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 mutation depends on the nature of the SD sequence of a given gene. The strong effect on \textit{pssA} translation reflects the suboptimal nature of the SD sequence of \textit{pssA}, which might be better recognized by the mutated anti-SD sequence of 16S RNA. This weak initiation of translation might be important in the control of \textit{pssA} expression and highlights the fact that gene expression can be modulated by other means than specific regulatory processes.
MATERIALS AND METHODS

Strain Constructions

For all strain and plasmid constructions, the Ecocyc website \(^{(21)}\) was used for sequence retrieval. The construction of the various strains is described succinctly in Table 2. Deletions of \(hdfR, rrnC, rrlC, \) or \(rrsC\) were performed by direct recombination of PCR fragments obtained using \(pKD3\) or \(pKD4\) plasmids as templates, following the Datsenko 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 \(^{(13)}\), were transduced from one background to another by generalized transduction with the phage P1 \(^{(24)}\).

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 \(^{(26)}\).
Measure of expression using transcriptional and translational fusions with GFP

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

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.

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,
sequencing grade). Resulting peptides were analyzed in duplicates by online nanoLC-MS/MS (UltiMate 3000 and LTQ-Orbitrap Velos Pro, Thermo Scientific) using a 120-min gradient. Peptides and proteins were identified and quantified using MaxQuant (version 1.5.7.4, (28)) and Uniprot database (February 2017 version, *Escherichia coli* K12 taxonomy). For statistical analysis, we used ProStaR (29): proteins identified in the reverse and contaminant databases and proteins exhibiting less than 4 intensity values in one condition were discarded from the list. After log2 transformation, intensity values were normalized by median centering before missing value imputation (replacing missing values by the 1 percentile value of each column); statistical testing was 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 Benjamini-Hochberg method.

**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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References


Table 1: Genome sequence differences between AC1 and AC5 strains identified by genome resequencing

a: Positions of the mutations on the *E. coli* K-12 MG1655 reference genome as given in Ecocyc database (21).

b: Missense mutations and the corresponding genes are highlighted in bold. *yifK* gene used for mapping pssR1 is also indicated.

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

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**Table 2: *E. coli* K-12 strains**

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<tr>
<td>EB944</td>
<td>MG1655</td>
<td>Wild type parental <em>E. coli</em> K-12 strain</td>
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<tr>
<td>EB801</td>
<td>BW25115 ΔhdfR::kanaR</td>
<td>Transduction ΔhdfR::kanaR from EB801 to MG1655; cassette removed using pCP20 plasmid</td>
<td>(29)</td>
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<tr>
<td>EB466</td>
<td>BW25113 ΔyifK::kanaR</td>
<td>This work</td>
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</tr>
<tr>
<td>EB814</td>
<td>MG1655 ΔhdfR</td>
<td>Transduction ΔhdfR::kanaR from EB801 to MG1655; cassette removed using pCP20 plasmid</td>
<td>This work</td>
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<tr>
<td>EB815</td>
<td>AC1</td>
<td>pssR+</td>
<td>(10)</td>
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<tr>
<td>EB816</td>
<td>AC5</td>
<td>pssR1</td>
<td>(10)</td>
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<tr>
<td>EB356</td>
<td>AC1 ΔhdfR::cat</td>
<td>PCR ebm1094/1081 on pKD3 of the cat cassette recombined in AC1</td>
<td>This work</td>
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<tr>
<td>EB886</td>
<td>AC5 ΔhdfR::cat</td>
<td>PCR ebm1094/1081 on pKD3 of the cat cassette recombined in AC5</td>
<td>This work</td>
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<td>EB1011</td>
<td>MGΔhdfR::cat pssR+</td>
<td>Transduction ΔhdfR::cat from EB356 to MG1655</td>
<td>This work</td>
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<tr>
<td>EB430</td>
<td>MGΔhdfR::cat pssR1</td>
<td>Transduction ΔhdfR::cat from EB886 to MG1655</td>
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<tr>
<td>EB1068</td>
<td>AC5ΔyifK::kanaR (JB70) pssR1, hdfRwt</td>
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<td>Collection of strains with −SPA or −TAP tags on the chromosome</td>
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<td>EB1032</td>
<td>AC1/PlsB-SPA</td>
<td>Transduction of plsB-SPA-kanaR from the −SPA collection to AC1</td>
<td>This work</td>
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<td>EB1033</td>
<td>AC5/PlsB-SPA</td>
<td>Transduction of plsB-SPA-kanaR from the −SPA collection to AC5</td>
<td>This work</td>
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<tr>
<td>EB1050</td>
<td>AC1/PlsC-SPA</td>
<td>Transduction of plsC-SPA-kanaR from the −SPA collection to AC1</td>
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<tr>
<td>EB1051</td>
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<tr>
<td>EB1054</td>
<td>AC1/PgsA-TAP</td>
<td>Transduction of pgsA-TAP-kanaR from the −SPA collection to AC1</td>
<td>This work</td>
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<tr>
<td>EB1055</td>
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<td>Transduction of pgsA-TAP-kanaR from the −SPA collection to AC5</td>
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<td>EB350</td>
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<td>PCR ebm1624/1620 on pKD4 recombined in AC1</td>
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<tr>
<td>EB352</td>
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</tr>
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<td>AC1ArrsC</td>
<td>PCR ebm1624/1640 on pKD4 recombined in AC1</td>
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<td>EB1063</td>
<td>AC5ArrsC</td>
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Table 3: Plasmids

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<td>pEB0269</td>
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<td>ampIR, FRT-kanaR-FRT cassette</td>
<td>(22)</td>
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<tr>
<td>pEB0267</td>
<td>pKDGT</td>
<td>Ts, ampIR, lambda Red genes</td>
<td>(22)</td>
</tr>
<tr>
<td>pEB0266</td>
<td>pCP20</td>
<td>Ts, ampIR, camR, FLP recombinase gene</td>
<td>(31)</td>
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<tr>
<td>pEB0227</td>
<td>pBAD24</td>
<td>ampIR, PBAD promoter</td>
<td>(32)</td>
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<td>pEB1408</td>
<td>pBAD-hd6R</td>
<td>PCR with ebm870/871 oligonucleotides on AC1 genomic DNA (EcoRI/XhoI) in pBAD24 (EcoRI/Sall)</td>
<td>This work</td>
</tr>
<tr>
<td>pEB1501</td>
<td>pBAD-hd6R(G237S)</td>
<td>PCR with ebm870/871 oligonucleotides on AC5 genomic DNA (EcoRI/XhoI) in pBAD24 (EcoRI/Sall)</td>
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<td>pEB0898</td>
<td>pUA66</td>
<td>kanaR, sc101 ori, GFPmut2</td>
<td>(25)</td>
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<td></td>
<td>pUA-UPyfE</td>
<td>Region -196/+99 relative to the start codon of yifE</td>
<td>(25)</td>
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<td>pEB1427</td>
<td>pTrad-GFPmut2</td>
<td>kanaR, sc101 ori, Δrbs, PCR mutagenesis on pUA66 with ebm927/928 oligonucleotides</td>
<td>This work</td>
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<td>pEB1611</td>
<td>pTrad-pssA(F1)</td>
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<tr>
<td>pEB1323</td>
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<td>pEB1900</td>
<td>pZE-Trad-GFPmut2</td>
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<td>pEB1864</td>
<td>pPsSsA mutant10</td>
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<td>pEB1878</td>
<td>pPsSsA mutSD</td>
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<td>kanaR</td>
<td>S. Quan, unpublished</td>
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<td>pEB1866</td>
<td>pcSacB-rrnC rrsC(C1538T)</td>
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a: Trad signifies translational fusion. b: Ts: thermosensitive replication; ori: origin of replication; Δrbs: deletion of ribosome binding site on the vector.
Figure legends

Figure 1: Upregulation of pssA in the pssR1 mutant. A. Strains AC1 (pssR+), AC5 (pssR), AC1ΔhdfR::camR (EB356); AC5ΔhdfR::camR (EB886), MGAΔhdfRpssR+ (EB1011), and MGAΔhdfRpssR1 (EB430) (Table 2) were transformed with the pssA-GFP translational fusion and the corresponding control (plasmids pEB1853 and pEB1900 respectively), and expression of pssA-GFP was measured as described in Materials and Methods. Data are the mean of four replicates each and the error bars show the standard deviations. B. Strains AC1 and AC5 producing tagged phospholipid enzymes, EB160 and EB161 (PssA-SPA), EB1052 and EB1053 (PlsB-SPA), EB1050 and EB1051 (PlsC-SPA), and EB1054 and EB1055 (PgsA-TAP) (Table 2), were grown in LB at 37°C and stopped in exponential growth phase. Whole cell extracts were separated on 10% SDS-PAGE and the SPA-tagged or TAP-tagged proteins were analyzed by Western blot using anti-Flag or PAP antibodies respectively. C. The experiment shown in B. was repeated with three or four replicates for each strain, and the Western blots were quantified on a Li-Cor Imager. The error bars show the standard deviations.

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 ΔhdfR (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* expression in AC5. **A. rrnC operon.** The positions of the mutations present in AC5 but not in AC1 are indicated in red. **B.** Strains AC1 or AC5 deleted of the portions of the *rrnC* operon indicated (strains EB350, EB349, EB1062, EB352, EB351, EB1063, see Table 2) were transformed with the *pssA*-GFP translational fusion (plasmid pEB1853), and expression was monitored as described in Materials and Methods. Data are the mean 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) and the indicated plasmids (cont: pEB0354; *prrnC*: pEB1858; *prrnC*(C1538T): pEB1866). Expression was monitored as described in Materials and Methods. Data are the mean of 4 replicates each and the error bars show the standard deviations.

**Figure 4**: *rrsC*(C1538T) mutation affects *pssA* translation initiation. **A.** 5'UTR sequence of *pssA* and wild type or mutated anti-SD sequences of 16S RNA are shown. Mutations are indicated in red. TSS: transcription start site. The different constructions of GFP fusions with portions of *pssA* gene are shown below. Regions coming from the *pssA* gene are drawn in black; Plac promoter is drawn in blue, and artificial 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 pEB1853, pEB1864, and pEB1878 (B); plasmids pEB1611, pEB1856, and pEB1895 (C)).
Expression was monitored as described in Materials and Methods. Data are the mean of 4 replicates each and the error bars show the standard deviations.
Figure 1

A

Fluorescence / OD_{600nm} (A.U.)

B

PssA-SPA
PIsB-SPA
PIsC-SPA
PgsA-TAP

pssR+  pssR1

C

Band intensity

pssR+  pssR1
Figure 2

A

![Western Blot Image]

B

**pTrad-pssA**

![Graph]

C

**pUA-yfE**

![Graph]
Figure 3

(A) Diagram of the gene locations:

- rrsC
- rrlC
- rrfC
- hdfR
- yifE

(B) Fluorescence/OD200m (A.U.) for AC1 and AC5:

- mC
- mtC
- rsC

(C) Fluorescence/OD200m (A.U.) for AC1 and AC5:

- cont
- pmC
- pmC(mt53T)

(insert descriptions and analysis if needed)
Figure 4:

A

S16 RNA wt  
3'AUUCCUCCACUAG 5'

S16 RNA mutC1538U  
{3'AUUCUCCACUAG 5'}
{3'AUUCUCCACUAG 5'}

start pssA

G (mutSD)

TSS

GGTTATATTCGGCTGCCTATGTCGTGATCATACAGAGGACCTTTCAATGAACAGAGAAGAATGCACCTGTGATG

CG (mut-10)
PpssA

pssA ORF

pssA-GFP fusion (F1)
PpssA fusion (F2)
Plac-S'UTRpssA fusion (F3)

B

C

Fluorescence / OD_{600nm} (A.U.)

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<td>AC1</td>
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<td>mut -10</td>
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<td>mut SD</td>
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<th>Fluorescence / OD_{600nm} (A.U.)</th>
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