Transcriptomic analysis of *Pseudomonas ogarae* F113 reveals the antagonistic roles of AmrZ and FleQ during rhizosphere adaption

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**Abstract**

Rhizosphere colonization by bacteria involves molecular and cellular mechanisms, such as motility and chemotaxis, biofilm formation, metabolic versatility, or biosynthesis of secondary metabolites, among others. Nonetheless, there is limited knowledge concerning the main regulatory factors that drive the rhizosphere colonization process. Here we show the importance of the AmrZ and FleQ transcription factors for adaption in the plant growth-promoting rhizobacterium (PGPR) and rhizosphere colonization model *Pseudomonas ogarae* F113. RNA-Seq analyses of *P. ogarae* F113 grown in liquid cultures either in exponential and stationary growth phase, and rhizosphere conditions, revealed that rhizosphere is a key driver of global changes in gene expression in this bacterium. Regarding the genetic background, this work has revealed that a mutation in *fleQ* causes considerably more alterations in the gene expression profile of this bacterium than a mutation in *amrZ* under rhizosphere conditions. The functional analysis has revealed that in *P. ogarae* F113, the transcription factors AmrZ and FleQ regulate genes involved in diverse bacterial functions. Notably, in the rhizosphere, these transcription factors antagonistically regulate genes related to motility, biofilm formation, nitrogen, sulfur, and amino acid metabolism, transport, signalling, and secretion, especially the type VI secretion systems. These results define the regulon of two important bifunctional transcriptional regulators in pseudomonads during the process of rhizosphere colonization.

**DATA SUMMARY**

The RNA-Seq data used throughout this study are publicly accessible through NCBI, under the BioProject PRJNA419480. BioSamples accessions: F113 culture in exponential (SAMN17839758 and SAMN17839763) and stationary (SAMN17839759 and SAMN17839764) growth phases or under rhizosphere conditions (SAMN17839757 and SAMN17839762); *amrZ* mutant (SAMN17839760 and SAMN17839765) and *fleQ* mutant (SAMN17839761 and SAMN17839766) grown under rhizosphere conditions.

Other supporting data are available as Supplementary Files in the online version of this article.

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.
Impact Statement

Bacteria possess diverse signal transduction systems that allow the integration of stimuli from the surrounding environment with cell responses, ultimately allowing their adaption to changing environments. This process is key for biotechnological applications such as the use of PGPRs as inoculants and future manipulation of crop microbiome for sustainable agriculture. In this regard, the transcription factors AmrZ and FleQ have been recently proposed as global regulators of gene expression in different Pseudomonas species. In the PGPR P. ogarae F113, AmrZ and FleQ constitute a transcriptional regulatory hub. Using transcriptomic analysis, we have determined that the AmrZ/FleQ hub acts also as a global regulatory node in the adaption to the rhizosphere environment of P. ogarae F113. The transcription factors AmrZ and FleQ work mainly in an antagonistic manner in the control of relevant functions during the process of rhizosphere colonization which include motility and chemotaxis, biofilm formation, bacterial secretion systems, ABC transporters, and the metabolism of nitrogen, amino acids, and sulfur. The results presented in this work have deepened in the molecular mechanisms involved in the bacterial colonization of a complex and changing environment such as the rhizosphere and could be relevant due to the ubiquitous nature of Pseudomonas species in the environment and their association with diverse hosts.

INTRODUCTION

Bacterial adaption to different environments requires the tight control of gene expression utilizing diverse transcriptional regulators. RNA polymerases interact with sigma factors to initiate transcription, which can, in turn, cooperate with different transcription factors (TFs) to coordinate the regulation of gene expression and bacterial behaviour. In this regard, AmrZ and FleQ are known regulators of diverse bacterial functions in pseudomonads [1, 2]. AmrZ is a ribbon-helix-helix TF that acts as a global and bi-functional regulator of gene expression in pseudomonads [3]. For instance, the RNA-Seq analysis of an amrZ mutant in P. aeruginosa showed that this TF controls motility, exopolysaccharide synthesis, and cyclic diguanylate (c-di-GMP) metabolism [4]. Specifically, AmrZ is a negative regulator of flagellar synthesis acting as a repressor of the transcription factor FleQ [5–7] and has also been related to the control of virulence [4, 8, 9]. AmrZ has been found to target similar functions in P. syringae, P. putida, and P. stutzeri [10–15].

On the other hand, FleQ belongs to the NtrC/NifA family and regulates the flagella biosynthesis and other mechanisms in several bacteria [16–18] and has been thoroughly studied in the Pseudomonas genus. In P. aeruginosa and P. syringae, FleQ is involved in the regulation of genes involved in flagellar and extracellular matrix synthesis [12, 16, 19]. Furthermore, FleQ was also found to regulate the type VI secretion system (T6SS) and c-di-GMP metabolism, as described in P. putida [20], and virulence, as shown in P. syringae [12]. Although FleQ has been identified as the transcriptional activator of flagellar synthesis and adhesion in strains belonging to the P. fluorescens group of species [21], including Pf0-1 and SBW25 [22–25], the role of FleQ in other functions are poorly studied in this group of bacteria.

In the model rhizosphere coloniser and PGPR, Pseudomonas ogarae F113 [26] (formerly Pseudomonas fluorescens F113), the direct regulons of AmrZ and FleQ on liquid media have previously been studied by Chromatin immunoprecipitation sequencing (ChIP-Seq) analyses [27] and different phenotypic assays have been carried out with both mutants [28, 29]. These works allowed the description of AmrZ as a global regulator of hundreds of genes, among which it acts as a negative regulator of motility, a positive regulator of biofilm formation, and is crucial for competitive rhizosphere colonization. ChIP-Seq analysis has shown that FleQ is also a global transcriptional regulator, which affects more than 120 genes, including motility, attachment, and homeostasis related genes [30]. This work also showed that AmrZ and FleQ share part of their regulons and it was proposed that both transcription factors act as a regulatory hub, which antagonistically regulate motility, exopolysaccharides production and iron homeostasis genes, in a c-di-GMP-dependent way.

One approach to tackle bacterial adaption to specific environments is studying the transcriptome in different environmental conditions and genetic backgrounds [31–34]. This work aims to analyse the transcriptomic profile of P. ogarae F113 and its isogenic mutants amrZ and fleQ, impaired in rhizosphere colonization [28, 29]. To address this, we have used the RNA-Seq technique in different bacterial growth conditions. The results provide hints about the molecular regulatory pathways of AmrZ and FleQ and bacterial functions involved in adaption mechanisms to the rhizosphere environment.

METHODS

Bacterial strains and growth conditions

Bacterial strains used in this work include the P. ogarae F113 wild-type strain [35], the amrZ and fleQ mutants previously constructed by homologous recombination [29, 30, 36]. Pseudomonas ogarae F113 and derivatives were routinely grown in Minimal Sucrose-Asparagine (SA) medium [37] at 28 °C with shaking. These mutants were previously phenotypically analysed...
and verified by complementation of at least the swimming motility phenotype [27–30, 36]. Prior RNA isolation of F113 and derivatives, swimming motility experiments were performed as a quality test. Swimming motility assays were done following the procedure detailed in [30].

**Bacterial RNA isolation from cultures**

RNA was isolated from F113 and derivatives cultures grown in SA medium [37] at exponential (Optical density (OD)₆₀₀ = 0.6) and stationary phase (OD₆₀₀ =1.2) [38]. All the steps of the RNA isolation were done at 4 °C except otherwise specified. Three independent cultures of each condition were mixed preceding RNA isolation and 15 ml of the sample mix were centrifuged at 4200 g for 5 min. Then, supernatants were discarded and pellets were frozen with liquid nitrogen and stored at −80 °C. Frozen pellets were resuspended in 200 µl of lysis buffer (Tris–HCl 1 M, EDTA 0.1 M pH 8, and 0.4 mg ml⁻¹ lysozyme) and incubated for 5 min at RT. Total RNA was extracted using the Trizol-chloroform method. Briefly, 1 ml of TRizol was added to each sample and incubated for 5 min at RT. Subsequently, 0.2 ml of chloroform was added, vigorously agitated, and incubated for 3 min at RT. Samples were centrifuged for 15 min at 12000 g. The aqueous phase was transferred to a clean tube and 0.5 ml of isopropanol added. Samples were then agitated by inversion and kept for 10 min and further centrifuged for 10 min at 12000 g. The supernatant was discarded and the RNA pellet was cleaned with 1 ml of 75% (v/v) ethanol. Cleaned samples were centrifuged 5 min at 7500 g and 2 min at 7500 g to assure complete ethanol removal. Samples were air-dried at RT for 5 min and resuspended in 20 µl of RNase-free water for 5 min at 4 °C. Subsequently, 1 µg of RNA was treated with DNase I (RQ1 Promega) following the instructions provided by the manufacturer and RNA Clean and Concentrator kit (Zymo Research), and finally resuspended in 15 µl of RNase-free water.

**Bacterial RNA isolation from alfalfa rhizosphere**

*Alfalfa (Medicago sativa var. Resis) seed* were surface-sterilized with 70% (v/v) ethanol for 2 min and 5% (v/v) sodium hypochlorite for 15 min. Then, seeds were thoroughly washed and germinated on sterile 1% (v/v) agar distilled water for 72 h in the dark at 28 °C. Seedlings were transferred into sterile 50 ml tubes containing 15 g pre-wetted medium-grain vermiculite and 5 ml Fähraeus Plant (FP) medium [39] and transferred to a plant growth chamber (16/8 h, light/dark photoperiod, and 25/18 °C). Thirty-two seedling-tubes were prepared per wild-type and derivatives to obtain a greater yield of isolated-RNA.

Strains were grown overnight in SA medium and diluted in FP to inoculate 1 ml of 1·10⁸ Colony forming Units (CFUs) at the base of each alfalfa 7 day-old seedling. RNA isolation was carried out 7 days post-inoculation [38]. After removing shoots of alfalfa plants, roots and vermiculite were placed on 10 ml of 60% (v/v) RNAlater [40] in pH 7.4 phosphate-buffered saline and vortexed for 2 min in order to resuspend bacterial cells. A mix of 32 preparations per condition was filtered using four layers of sterile muslin cloth. The recovered filtrate was centrifuged for 1 min at 200 g and 4 °C. Then, the supernatant was transferred and centrifuged again at 4200 g for 20 min and 4 °C. Supernatants were discarded and pellets dried before liquid nitrogen freezing. Frozen pellets were stored until use. RNA extraction was performed as described in the previous subsection.

**RNA sequencing, bioinformatic analysis, and data processing**

The Qubit fluorometer quality assessment, rRNA depletion procedure, the strand-specific library construction, and sequencing were performed by Novogene Co., Ltd. (Beijing, China, and Cambridge, UK) using Illumina HiSeq paired-end, 2×150 bp [38].

A new annotation for *P. ogarae* F113 was generated using Prokka software version 1.14.5 [41]. The annotation in the Pseudomonas Genome Database [42] was compared to the new one using Blast [43] to keep the old locus annotation and to add the identifiers to the newfound genes. All coding DNA sequences (CDSs) were further annotated using eggNOG-mapper v1.0.3 [44] to get Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) codes.

After RNA sequencing, reads were trimmed and quality-filtered using Trimmomatic v 0.38 [45], specifying a four nts sliding window with an average phred quality of 15 and 50 nts as minimum read length as described in [38]. Then, trimmed reads were randomly subsampled into two technical replicates with 1000000 reads each using an own designed Python script.

At this point, high-quality reads were, on the one hand, used for operon prediction using the software Rockhopper version 2.0.3 [46, 47] from the *P. ogarae* F113 wild-type RNA-Seq data under culture and rhizosphere conditions. Operons were identified using a naive Bayes classifier based on prior operon probabilities, intergenic distance, and gene expression correlation across RNA-Seq experiments. On the other hand, high-quality reads were directly used for transcripts quantification using Salmon software version 1.3.0 [48] and the new annotation of the *P. ogarae* F113 CDSs as a reference (GenBank: NC_016830). Normalization of transcript counts using the median of ratios method and differential gene expression was calculated using *DESeq2* 1.24.0 R package [49]. Normalized data was used to check gene expression and compare the gene expression profile in every condition via the Principal Component Analysis (PCA) using *vsn* version 3.54.0 and *DESeq2* version 1.24.0 R packages [49, 50] and the cluster calculation based on Euclidean sample-to-sample distances using *DESeq2*.

Differential gene expression comparisons were made using the ‘contrast’ function of *DESeq2*, taking wild-type under rhizosphere condition as the reference, and setting a threshold for log₂ Fold-Change (wild-type under liquid culture conditions or mutant
versus wild-type under rhizosphere condition) of $-1 \leq 1$ and a stringent p-adjusted value cutoff $\leq 0.001$. Thus, positive Fold-Change values correspond to up-regulation on the wild-type grown under culture conditions or the mutant, whereas negative Fold-Change values indicate down-regulation.

Functional analysis of differentially expressed genes was made using ‘Biological process’ terms from the GO database [51] and the KEGG functional categories from the KEGG pathway database [52]. GO term enrichment was calculated using topGO R package version 2.42.0 with annotations made via eggNOG-mapper and a modified Fisher’s exact p-value [53]. KEGG categories were extracted from annotations in the KEGG database. KEGG pathway abundance was calculated as the ratio of differentially expressed genes/annotated genes per category.

PCA distance map and dot plots for enrichment were represented with ggplot2 R package version 3.3.2 [54]. Heatmap of the sample-to-sample Euclidean distances and heatmap of differential gene expression were made using pheatmap R package version 1.0.12 [55]. UpSet plots were made using UpSetR package 1.4.0 [56].

RESULTS AND DISCUSSION

Global analysis of the Pseudomonas ogarae F113 transcriptome

In this study, we analysed the transcriptome of P. ogarae F113 and derivatives in different growth conditions: liquid cultures of the wild-type strain at exponential and stationary phase in SA medium [37] and the wild-type strain and the amrZ and fleQ mutants after a week of growth in the rhizosphere of alfalfa (Medicago sativa) plants. Rhizosphere constitutes the most common ecological niche in which F113 is known to establish beneficial interactions with plants [57] and, therefore, could provide valuable information in the regulatory pathways involved in the bacterial adaption to the rhizosphere environment. To obtain the samples from the rhizosphere of alfalfa, we used artificial soil based on vermiculite that allowed us to exclusively trace the bacterial response to the presence of the plant.

After RNA Illumina HiSeq sequencing and quality filtering, we obtained ca. 20 million reads in each condition (File S1, available in the online version of this article). RNA-Seq data corresponding to Pseudomonas ogarae F113 wild-type grown in liquid culture at exponential and stationary phases or in the rhizosphere were used to identify its transcriptional organization with Rockhopper [46]. As a result, mapping against the F113 genome revealed 6064 CDSs organized in 2314 monocistronic operons and 1232 polycistronic operons (File S2). Polycistronic operones include more than half of the CDSs (3750), have an average length of 3068 nts, three CDSs per operon, and are equally distributed in both strands of the F113 chromosome (File S2).

Subsequently, reads were pseudo-mapped against F113 CDSs using Salmon [48] to quantify gene expression, resulting in a 50–70% mapping read rate (File S1). Global gene expression patterns in all the conditions were analysed via the calculation of the Principal Component Analysis (PCA, Fig. 1a) and the sample-to-sample distances (Fig. 1b). In Fig. 1(a), the first principal component (PC1), explaining the 82% of the observed variance, separates the transciptomes corresponding to liquid media cultures from those grown in the rhizosphere. The second principal component (PC2), which explains only the 10% of the observed variance, separates fleQ from the wild-type and the amrZ mutant in the rhizospheric samples. Thus, the PCA of the transcription profiles is able to separate samples into two groups: wild-type in liquid culture (1) and wild-type and both amrZ and fleQ mutants in the rhizosphere (2). This result demonstrates that growth in the rhizosphere is a main driver in the transcriptional profile of P. ogarae F113.

The differences observed in the PCA are further supported by the hierarchical Euclidean clustering (Fig. 1b) that shows the calculation of sample-to-sample distances. According to this clustering analysis, all the rhizospheric samples clustered together, being the wild-type background in an intermediate position between fleQ and amrZ mutant backgrounds. Moreover, the wild-type and amrZ samples were the closest ones in the rhizosphere, while fleQ clustered separately.

The results obtained from these analyses indicate that growth of F113 in the rhizosphere implies a transcriptional profile very different from that present during growth in liquid cultures. This observation agrees with the need for a tighter regulation to rapidly sense and respond in complex environments such as the rhizosphere [58, 59]. Moreover, our results show that the mutation in fleQ has a greater impact on gene expression than the amrZ mutation in the rhizosphere. Considering that the rhizosphere is the natural habitat of P. ogarae F113, we decided to focus on the differential gene expression and functional differences taking place under rhizosphere conditions between F113 and derivatives.

Differential gene expression analysis

The overall results of the comparison between F113 wild-type grown in the rhizosphere versus liquid cultures and the comparisons of the amrZ and fleQ mutants versus F113 wild-type under rhizosphere conditions are listed in File S3. Overall, 979 differentially expressed genes (DEGs) were identified above the set threshold of log, Fold-Change $-1 \geq 1$ and a p-adjusted value $\leq 0.001$ in all the studied conditions, from which 535 were up-regulated and 536 down-regulated in at least one of the comparisons.
Fig. 2(a) shows the number of DEGs found in F113 wild-type grown under liquid culture conditions versus rhizosphere conditions. The wild-type strain under rhizosphere conditions shows a distinct transcriptional profile from that observed in liquid cultures independently of the growth phase, with nearly 3000 differentially expressed genes in each liquid culture sample compared to the rhizosphere sample (Fig. 2a). In fact, as shown in Fig. 2(a) there is a large overlap in the intersection between up- or down-regulated genes in the exponential and stationary growth phases when these samples are compared to the sample grown under rhizosphere conditions.

Fig. 2(b) summarizes the number of DEGs found in amrZ and fleQ mutants compared to F113 wild-type under rhizosphere conditions. According to Fig. 2(b), 343 and 262 up-regulated and 144 and 460 down-regulated genes were found in the amrZ and fleQ mutants, respectively.
Fig. 2. Differential gene expression in *P. ogarae* F113 and derivatives in different growth conditions. Upset plot showing the intersections of shared and specific up- and down-regulated DEGs among *P. ogarae* F113 grown under culture conditions (a) and the *amrZ* and *fleQ* mutants under rhizosphere conditions (b). DEGs were calculated using F113 wild-type under rhizosphere condition as a reference. DEGs were selected applying the following threshold: log2 Fold-Change ≤−1/≥1 and a p-adjusted value ≤ 0.001. Set size indicates the total number of DEGs found in each condition. Exp: exponential; St: stationary.
and fleQ mutants, respectively in the rhizosphere. These results suggest that the role of AmrZ and FleQ in the rhizosphere are predominantly as a repressor and activator, respectively.

The comparison of the RNA-Seq data from this study with the ChIP-Seq previously published for these transcription factors in P. ochraceae F113 [27, 30] revealed that 25 genes showing differential expression in the amrZ mutant under rhizosphere conditions were also found in ChIP-Seq studies as containing an AmrZ-binding site [27]. Similarly, 44 genes that are differentially expressed in the fleQ mutant under rhizosphere conditions also contain a FleQ-binding site in their promoter region [30], suggesting mainly an indirect regulatory role for these transcription factors. However, deeper study is needed to elucidate if the gene regulatory network is controlled either directly or indirectly by AmrZ and FleQ.

The set of DEGs obtained for amrZ and fleQ mutants were compared (Fig. 2b). As a result, a large amount of DEGs is shared among the AmrZ and FleQ regulons suggesting similarities in the transcriptional profile of the amrZ and fleQ mutants. Nevertheless, the largest intersection of AmrZ and FleQ regulons was observed between the up-regulated DEGs in the amrZ mutant and the down-regulated DEGs in the fleQ mutant.

To observe those genes whose transcription is more dramatically affected in the F113 amrZ and fleQ mutants, the top ten up- and down-regulated DEGs under each condition were studied and listed in Table 1. These listed genes show that among

| Locus | Log_{FC} | Product | Locus | Log_{FC} | Product |
|-------|----------|---------|-------|----------|---------|
| 2459  | 5        | PhlD    | 455a  | 6.4      | HP      |
| 261   | 4.5      | Methionine ABC transporter ATP-binding protein | 2779  | 5.3      | Outer membrane autotransporter |
| 2460  | 4.3      | PhlB    | 1970  | 5.1      | PapA    |
| 5631  | 4.2      | SsuC    | 1968  | 5.1      | PapC    |
| 2461  | 4.2      | PhlC    | 1967  | 4.9      | PapD    |
| 0263  | 4.1      | DszA    | 1966  | 4.9      | Putative polysaccharide biosynthesis protein |
| 0264  | 4        | Acyl-CoA dehydrogenase | 2082  | 4.8      | YaeT    |
| 4855  | 4        | Putative translation initiation inhibitor, YigF family | 3004  | 4.6      | PsmE    |
| 5630  | 4        | SsuB    | 1969  | 4.5      | PapB    |
| 5075  | 3.9      | Catalase | 1965  | 4.3      | PapE    |
| 3338  | −4.9     | HP      | 3765  | −7       | NorA    |
| 2684  | −4.7     | FapB    | 1554  | −6.8     | FliC    |
| 2683  | −3.4     | FapC    | 1541  | −6.8     | dTDP-4-dehydrorhamnose 3,5-epimerase |
| 4192  | −3.1     | Flp-1   | 1560  | −6.6     | FleS    |
| 4186  | −2.9     | HP      | 1562  | −6.4     | FliE    |
| 2682  | −2.7     | FapD    | 2014  | −6.3     | CapG    |
| 2681  | −2.7     | FapE    | 1561  | −6.2     | FleR    |
| 4187  | −2.6     | Von Willebrand factor type A domain protein, associated with Flp pilus assembly | 1539  | −6.2     | Glucose-1-phosphate cytidylyltransferase |
| 0161  | −2.4     | PgaA    | 1536  | −6.2     | FlgK    |
| 4190  | −2.4     | TadV    | 4454  | −6.2     | FlgB    |

HP, hypothetical protein
them, many correspond to genes that encode proteins related to flagellar synthesis and function, chemotaxis, and the synthesis of extracellular matrix (ECM) components. Motility related DEGs include genes encoding FliC, MotY, Flg and Fle proteins, and methyl-accepting chemotaxis proteins. On the other hand, the DEGs related to ECM components include genes encoding the synthesis of the *Pseudomonas* acidic polysaccharide (Pap; *pap* genes) [60], poly-β−1,6-N-acetylglucosamine (PNAG; *pga* genes), functional amyloids in *Pseudomonas* (Fap; *fap* genes), the PsmE putative adhesin, and the fimbrial low molecular weight/tight adherence (Flp/Tad) pilus proteins. Interestingly, according to these results, AmrZ and FleQ have an opposite role regulating flagellar and ECM components synthesis, being AmrZ predominantly a positive regulator of biofilm-related genes and repressor of motility-related genes, in agreement with a previous study under liquid medium growth conditions [28]. In contrast, FleQ could have the opposite role controlling these functions.

Additionally, the genes *phlC* and *phlD*, which are part of the *phlACBD* operon encoding the components involved in the synthesis of the antifungal compound 2,4-diacetylphloroglucinol (DAPG) the most important biocontrol trait in F113 [35], were among the genes that appeared more differentially expressed under the studied conditions, indicating a role for AmrZ and FleQ in the regulation of this trait in F113. DAPG synthesis is subjected to regulation by different sigma and transcription factors in pseudomonads [61], and this list should be expanded to include these two transcriptional regulators.

**Functional analysis of DEGs**

In this study, we used two different methods of functional analysis to comprehensively understand the behaviour of AmrZ and FleQ TFs during the adaption to the rhizosphere environment: the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [52] and the Gene Ontology (GO) terms [51]. The KEGG categories (Fig. 3a) and GO terms (Fig. 3b) assigned to annotated DEGs were analysed to provide information on the proportion of regulated genes belonging to a particular functional category relative to the proportion of annotated genes in that category. The KEGG pathways containing more than two annotated DEGs were selected. Fig. 3(a) shows that the pathways affected under rhizosphere conditions in the *amrZ* and *fleQ* mutants are metabolic pathways, especially of nitrogen, amino acids and sulfur, ATP-binding cassette (ABC) transporters, bacterial secretion
systems, and biofilm and motility related pathways. A similar observation was made mapping DEGs through the GO database and selecting the top ten most representative GO terms (Fig. 3b), highlighting the abundance of enriched categories related to motility and chemotaxis, adhesion, and metabolism.

Moreover, the KEGG pathway analysis of the set of DEGs that is shared between the amrZ and fleQ mutants (according to Fig. 2b) revealed that AmrZ and FleQ affect in a similar way the expression of genes related to sulfur metabolism, ABC transporters, purine metabolism, biosynthesis of secondary metabolites, flagellar assembly, O-antigen nucleotide sugar biosynthesis, biosynthesis of nucleotide sugars and amino sugar and nucleotide sugar metabolism. On the other hand, both transcription factors affect oppositely genes related to the KEGG pathways of biofilm formation, bacterial secretion system, nitrogen metabolism, bacterial chemotaxis, quorum sensing, two-component system, microbial metabolism in diverse environments, and biosynthesis of secondary metabolites.

Recent studies have demonstrated that AmrZ is a global regulator in different pseudomonads. Using the RNA-Seq and ChIP-Seq approaches, Jones et al. (2014) found that, in Pseudomonas aeruginosa, AmrZ regulates virulence, aggregation, flagellum synthesis, chemotaxis, multidrug transport, rhamnolipid production, c-di-GMP signalling, and quorum sensing [4]. Furthermore, a ChIP-Seq study in F113 revealed that AmrZ is a regulator of environmental adaption that binds to the promoter region of hundreds of genes with a variety of functions, such as motility and chemotaxis, signal transduction pathways including c-di-GMP signalling, and iron homeostasis [27, 28]. Similarly, the TF FleQ, although initially known for its role in controlling flagellar synthesis, has also been related to a broader array of bacterial functions as described in Xanthomonas oryzae pv. oryzae, including flagellar-dependent and twitching motilities, virulence, exopolysaccharide production, biofilm formation, and siderophore production [18]. In pseudomonads, FleQ is also regulating biofilm formation in different species [12, 62–67] and the T6SS in P. putida [20], including P. orae F113 [30, 38].

Fig. 3 also suggests that AmrZ works mainly as a negative transcriptional regulator when grown in the rhizosphere, while FleQ acts similarly as a positive and negative regulator under this condition. Jones et al. (2014) [4] also demonstrated that AmrZ functions predominantly as a negative regulator in P. aeruginosa with the finding of 89 activated genes and 249 repressed genes by this TF [4]. Regarding FleQ, Xiao et al. (2021) [68] showed a similar number of up- and down-regulated genes, 172 and 272 respectively, in a fleQ mutant in P. putida compared to the wild-type strain. In agreement with this work, a significant number of those genes were related to cell motility and secretion processes, defence mechanisms, amino acid transport/metabolism, carbohydrate transport/metabolism, and signal transduction [68].

Given the number of genes affected by AmrZ and FleQ, we will focus in the following sections on those DEG categories with more relevance during the rhizosphere colonization process and that are among the DEGs with more significant values of differential expression according to Fig. 4(a).

**Biofilm formation**

Biofilm formation is an essential process during bacteria-host interaction and persistence to harsh conditions [69, 70]. Its relevance across the Pseudomonas genus is still being studied. Whereas it is crucial for rhizosphere colonization in certain species, it has a dispensable role in F113 [71, 72]. In this study, the functional analysis of RNA-Seq data using KEGG and GO databases revealed that biofilm formation is one of the most represented categories in the amrZ and fleQ mutants under rhizosphere conditions (Fig. 3a, b). As shown in Fig. 4(b) a remarkable finding is that, in the rhizosphere, AmrZ and FleQ are acting as antagonists in the regulation of the *flp/tad* and *papA-P* clusters, encoding a type IV pili and putative polysaccharide respectively. These two biofilm-related components could have a role during rhizosphere colonization. For instance, the Flp/Tad pilus was already identified as a key component in plant colonization [73]. RNA-Seq analysis in *P. aeruginosa* also showed that AmrZ is a regulator of polysaccharides such as alginate and *pel* clusters that affects the polysaccharide profile [4]. Similarly, FleQ has been associated with the regulation of the Psl and Pel polysaccharides in *P. aeruginosa* [19] and cellulose in *P. syringae* [12].

**Motility and chemotaxis**

As previously mentioned, one of the most affected group of genes in the transcriptomic comparison of *fleQ* and *amrZ* under rhizosphere conditions were those involved in the flagella biosynthesis and chemotaxis. The ability to sense and respond to root exudates is crucial during rhizosphere colonization in this bacterium [29, 71, 74, 75]. Flagellar synthesis and chemotaxis are two highly energy-consuming processes [76] and, therefore, are tightly regulated.

According to KEGG and GO functional analyses (Fig. 3), certain genes related to motility were found down-regulated in the *amrZ* mutant compared to the wild-type in the rhizosphere despite the known role of AmrZ as a negative regulator of motility. Nevertheless, in both the wild-type and the *amrZ* mutant, flagellar genes were expressed under rhizosphere conditions, evidenced by the high number of normalized counts in both cases (File S4), suggesting that motility is also essential for the wild-type in the rhizosphere. Nonetheless, chemotaxis is still found among the most relevant categories in the up-regulated genes in the *amrZ* mutant (Fig. 3). On the other hand, the role of FleQ as a positive regulator of motility is observed under rhizosphere growth conditions (Fig. 3). Our results are concordant with previous studies [27, 28, 36] which show that *fleQ* mutants are aflagellated.
Fig. 4. Differential gene expression of relevant pathways during the rhizosphere colonization process in the amrZ and fleQ mutants. (a) Volcano plot representing the differential gene expression analysis in the amrZ and fleQ mutant data sets versus wild-type under rhizosphere conditions. The x-axis shows log2 Fold-Change (FC) expression and the y-axis the odds of a gene being differentially expressed as the -log10(P-adjusted value). Black lined shapes indicate whether genes meet the following threshold: log2 FC ≤−1/≥1 and a P-adjusted value ≤0.001 whereas grey points indicate genes that do not meet either of the two criteria. Distinct point shapes indicate differentially expressed genes in one of the mutants versus the wild-type strain belonging to a relevant functional pathway: biofilm formation, nitrogen metabolism, c-di-GMP, motility, chemotaxis and T6SS. (b) Heatmap representation of genes belonging to relevant functional pathways in the amrZ and fleQ mutants in the rhizosphere. Pseudomonas ogarae F113 annotated genes with a log2FC (mutant/wild-type) ≤−1/≥1 and belonging to the KEGG database categories specified above are represented. The gene name or locus (‘PSF113_’) is shown. Values, locus, and annotations are listed in File S3.
Heatmaps for the specific DEGs in this category are shown in Fig. 4(b), revealing mainly up-regulation in the *amrZ* mutant and down-regulation in the *fleQ* mutant of genes encoding components of the second flagellar apparatus (left panel) in accordance with the proposed model of regulation in this bacterium [77] although controlling mostly a different set of genes. Moreover, Fig. 4(b) shows a predominant down-regulation of genes encoding components and regulators of the main flagellar apparatus (right panel) in the *fleQ* mutant.

**Bacterial secretion systems**

As shown in Fig. 3, AmrZ and FleQ act antagonistically in the regulation of the bacterial secretion systems in F113. Bacterial secretion systems allow the secretion or translocation of proteins useful for biocontrol properties as they intervene in rhizosphere colonization and the competition with eukaryotic and prokaryotic organisms [78]. We have previously shown that AmrZ and FleQ regulate T6SSs in the rhizosphere, being AmrZ and FleQ a negative and positive regulator respectively for F1-T6SS and F3-T6SS [38]. Bacterial T6SSs perform different activities, being the most relevant its role as a weapon that injects toxins into eukaryotic and prokaryotic cells, but also biofilm formation and regulation [79].

AmrZ and FleQ have been previously described as regulators of the T6SSs in the *Pseudomonas* genus. AmrZ is a direct regulator of T6SS in *P. aeruginosa* involved in pathogenicity [4, 9, 80], acting as a positive regulator of H1- and H3-T6SSs and negative regulator of H2-T6SS. On the other hand, FleQ is a negative regulator of the K1-T6SS through c-di-GMP in *P. putida* KT2442 [20]. The functional plasticity of AmrZ and FleQ, working as activators or repressors depending on intracellular second messengers or bacterial species, was described for motility and biofilm formation [12, 14, 62–67].

**Nitrogen metabolism**

The nitrogen metabolism pathway is also affected by the mutation of *amrZ* and *fleQ* under rhizosphere conditions (Fig. 3). Nitrogen metabolism is important in the rhizosphere [21, 81], in which it is found in the form of nitrate and ammonia.

As shown in Fig. 4(b), the nitrate reductase (*nar* and *nap*), nitrite reductase (*nir*), and the nitric oxide metabolism (*nos*) genes were up-regulated in the *amrZ* mutant and down-regulated in the *fleQ* mutant compared to the wild-type strain, indicating that while FleQ activates denitrification in the rhizosphere, AmrZ represses this pathway. Denitrification can be an advantage for allowing growth under limited oxygen availability, which may occur in the rhizosphere, especially after flooding. We have previously shown that under anaerobic conditions, both nitrate and nitrite are used by F113 as electron acceptors [75]. Furthermore, a battery of nitrogen-related genes not affected by AmrZ was found down-regulated in the *fleQ* mutant, such as a *glnA* encoding a protein involved in ammonia assimilation via the glutamate/glutamine cycle and *nas* genes encoding proteins involved in nitrate assimilation (Fig. 4b).

The down-regulation of nitrogen metabolism observed in the *fleQ* mutant could be related to a secondary mutation in *ntrC* observed in *P. fluorescens* subjected to a strong selection towards motility. Taylor et al. (2015) [82] demonstrated that after 96 h, *fleQ* mutants under a strong selection for motility regained flagella. This regulatory rewire is caused by mutations in the NtrB/ NtrC two-component system involved in nitrogen uptake and metabolism. NtrC is a distant homolog of FleQ that can control the FleQ regulon under selective pressure, resulting in the disruption of nitrogen uptake and assimilation under those circumstances [82]. The rhizosphere is an environment that exerts a strong selection over hypermotile variants [71, 83]. Two possible scenarios that could explain the result observed during growth in the rhizosphere are the disruption of nitrogen metabolism due to NtrB/C secondary mutations or the role of FleQ in the cross-regulation of motility and nitrogen metabolism in F113. Concerning this last point, RpoN was initially described as the regulator of nitrogen metabolism and nitrogen fixation [84], but since then, several TFs such as AmrZ, FleQ, GacA, or ExsA, have been found as co-regulators in *P. aeruginosa* [1]. This study evidences the functional crosstalk between different global TFs to co-regulate their downstream genes in a complex manner. Specifically, Huang et al. (2019) [1] described AmrZ and FleQ as regulators of some *nos* and *nar* genes [1], in agreement with the results observed in this study (Fig. 4b).

**c-di-GMP metabolism**

A previous RNA-Seq analysis in the F113 *amrZ* mutant compared to the wild-type strain grown in liquid cultures showed the role of AmrZ in the transcriptional regulation of 36 genes encoding enzymes involved in the metabolism of the second messenger c-di-GMP [28]. Here we studied the regulation of the 45 c-di-GMP metabolic enzymes encoded in F113 by AmrZ and FleQ in the rhizosphere. As a result, 13 genes encoding c-di-GMP metabolic enzymes were differentially expressed in the *amrZ* or *fleQ* mutants (Fig. 4b), showing a predominant down-regulation of genes encoding proteins with a GGDEF or GGDEF and EAL domains in the *fleQ* mutant and up-regulation of genes encoding GGDEF and EAL domain-containing proteins in the *amrZ* mutant. Proteins containing a GGDEF domain are associated with the diguanylate cyclase activity necessary to synthesize the second messenger c-di-GMP. On the contrary, the EAL domain is present in phosphodiesterases, which catalyse the degradation of c-di-GMP [85]. The number of genes encoding diguanylate cyclases or phosphodiesterases that are differentially expressed in the *amrZ* mutant is considerably lower in the rhizosphere than in liquid cultures. Previous research showed that in *P. fluorescens*,...
there is a coordinate cascade of c-di-GMP signalling that implies the expression of different diguanylate cyclases and phosphodiesterases depending on the stage of rhizosphere colonization [86] that could explain the reduced number of DEGs in relation with c-di-GMP under these circumstances.

Other functions

The pathways related to the biosynthesis and metabolism of the three aromatic amino acids and the uncharged amino acids glycine, serine, and threonine were up-regulated in fleQ in the rhizosphere. In contrast, the metabolism of the charged amino acid arginine was found down-regulated in the fleQ mutant (Fig. 3). On the other hand, cyanomino acid metabolism was the only pathway regarding amino acid metabolism up-regulated in the amrZ mutant. The rhizosphere is characterized by the secretion of exudates by plant roots that consist mainly of organic acids, sugars, and amino acids, making amino acid metabolism an important pathway during rhizosphere colonization [87]. The ability to take up and use amino acids as a nitrogen source could confer a selective advantage in certain environments [87].

On the other hand, sulfur metabolism appeared as up-regulated in both amrZ and fleQ mutants in the rhizosphere (Fig. 3). Sulfur and amino acid metabolism can be modified depending on iron acquisition, as shown for P. aeruginosa under iron starvation conditions. It is altered as a compensation mechanism for the downregulation of iron-rich metabolic pathways such as the tricarboxylic acid (TCA) cycle enzymes [88]. It was previously demonstrated that AmrZ is a repressor of iron homeostasis in F113 [27] and FleQ is an activator of this function [30]. Thus, suggesting that the observed regulation of sulfur and amino acid metabolism pathways could be due to alterations in the iron homeostasis in the amrZ and fleQ mutants and that these TFs are involved in regulating these linked metabolic pathways. Heatmaps for the specific DEGs in this category are shown in File S5.

ABC transporter proteins are one of the largest protein superfamilies involved in transporting vital molecules and mediating bacteria-host interaction, niche colonization, and virulence [89–91]. In the rhizosphere, F113 encounters a complex environment that requires higher levels of expression of genes involved in nutrient acquisition or transport [92, 93]. Several ABC transport proteins were up- or down-regulated in amrZ and fleQ mutants (Fig. 3), most notably up-regulation of sulfur (cys) and sulfinate (ssu) ABC transporters in the amrZ mutant and down-regulation of urea transport (urt) and pvdE involved in iron homeostasis in the fleQ mutant (File S6). This finding highlights the role of both TFs in controlling the expression of nutrient metabolic and transport systems.

CONCLUSIONS

The comparative transcriptomic analyses of P. orarae F113 grown in liquid medium cultures or the rhizosphere of alfalfa plants reveal that the rhizosphere is a major driver of transcriptional changes in this bacterium. Furthermore, transcriptomic analyses in P. orarae F113 mutants affected in the genes amrZ and fleQ under rhizosphere conditions showed that the fleQ mutation has a more significant impact on the transcription profile of F113 than the mutation in amrZ. In this work, we observed changes in genes related to lifestyle transition and adaption to the plant environment. In general, the transcriptional profiles for the mutants affected in amrZ and fleQ genes reflect alterations of bacterial function and physiological state in the rhizosphere. Furthermore, this study shows the role of AmrZ and FleQ in the regulation of motility, biofilm formation, bacterial secretion systems, nitrogen, sulfur, and amino acids metabolism, and transport, among others, in the rhizosphere. The regulation of genes belonging to these functional categories by AmrZ and FleQ occurs mostly in an antagonistic manner. The results obtained in this work can provide useful information on the molecular cues underlying PGPR adaption mechanisms to the rhizosphere environment, which are important for an effective colonization.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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