MICROBIAL GENOMICS

RESEARCH ARTICLE

Blanco-Romero *et al.*, *Microbial Genomics* 2022;8:000750

DOI 10.1099/mgen.0.000750

MICROBIOLOGY SOCIETY

OPEN DATA OPEN ACCESS

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

Esther Blanco-Romero[1], David Durán[1], Daniel Garrido-Sanz[1,2], Rafael Rivilla[1], Marta Martín[1] and Miguel Redondo-Nieto[1,*]

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.

Received 09 September 2021; Accepted 25 November 2021; Published 11 January 2022

Author affiliations: [1]Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Darwin 2, 28049 Madrid, Spain; [2]Department of Fundamental Microbiology, University of Lausanne, CH-1015 Lausanne, Switzerland.

***Correspondence:** Miguel Redondo-Nieto, miguel.redondo@uam.es

Keywords: *Pseudomonas ogarae*; transcriptomics; rhizosphere; regulation; AmrZ; FleQ.

Abbreviations: ABC, ATP-binding cassette; c-di-GMP, cyclic diguanylate; CDS, coding DNA sequence; CFU, colony forming unit; ChIP-Seq, chromatin immunoprecipitation sequencing; DAPG, 2,4-diacetylphloroglucinol; DEG, differentially expressed gene; ECM, extracellular matrix; Exp, exponential; Fap, functional amyloids in pseudomonas; FC, fold-change; Flp/Tad, fimbrial low molecular weight/tight adherence; FP, fåhraeus plant; GO, gene ontology; HP, hypothetical protein; KEGG, kyoto encyclopedia of genes and genomes; OD, optical density; Pap, pseudomonas acidic polysaccharide; PCA, principal component analysis; PGPR, plant growth-promoting rhizobacterium; PNAG, poly-β–1, 6-N-Acetylglucosamine; RNA-Seq, RNA sequencing; RT, room temperature; SA, sucrose asparagine; St, stationary; TCA, tricarboxylic acid; TF, transcription factor; T6SS, type VI secretion system; WT, wild-type.

The transcriptome sequences of Pseudomonas ogarae F113 and derivatives used in this study are publicly available at the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA419480: P. ogarae F113 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.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Two supplementary files containing six supplementary figures and tables are available in the online version of this article.

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 (OD_{600}=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) seeds 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% (w/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 F*å*hraeus 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 $1{\cdot}10^8$ 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 10000000 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 $\log_2$ Fold-Change (wild-type under liquid culture conditions or mutant

versus wild-type under rhizosphere condition) of ≤−1/≥1 and a stringent p-adjusted value cutoff ≤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 operons 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 transcriptomes 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 $\log_2$ Fold-Change ≤−1/≥1 and a p-adjusted value ≤0.001 in all the studied conditions, from which 535 were up-regulated and 536 down-regulated in at least one of the comparisons.

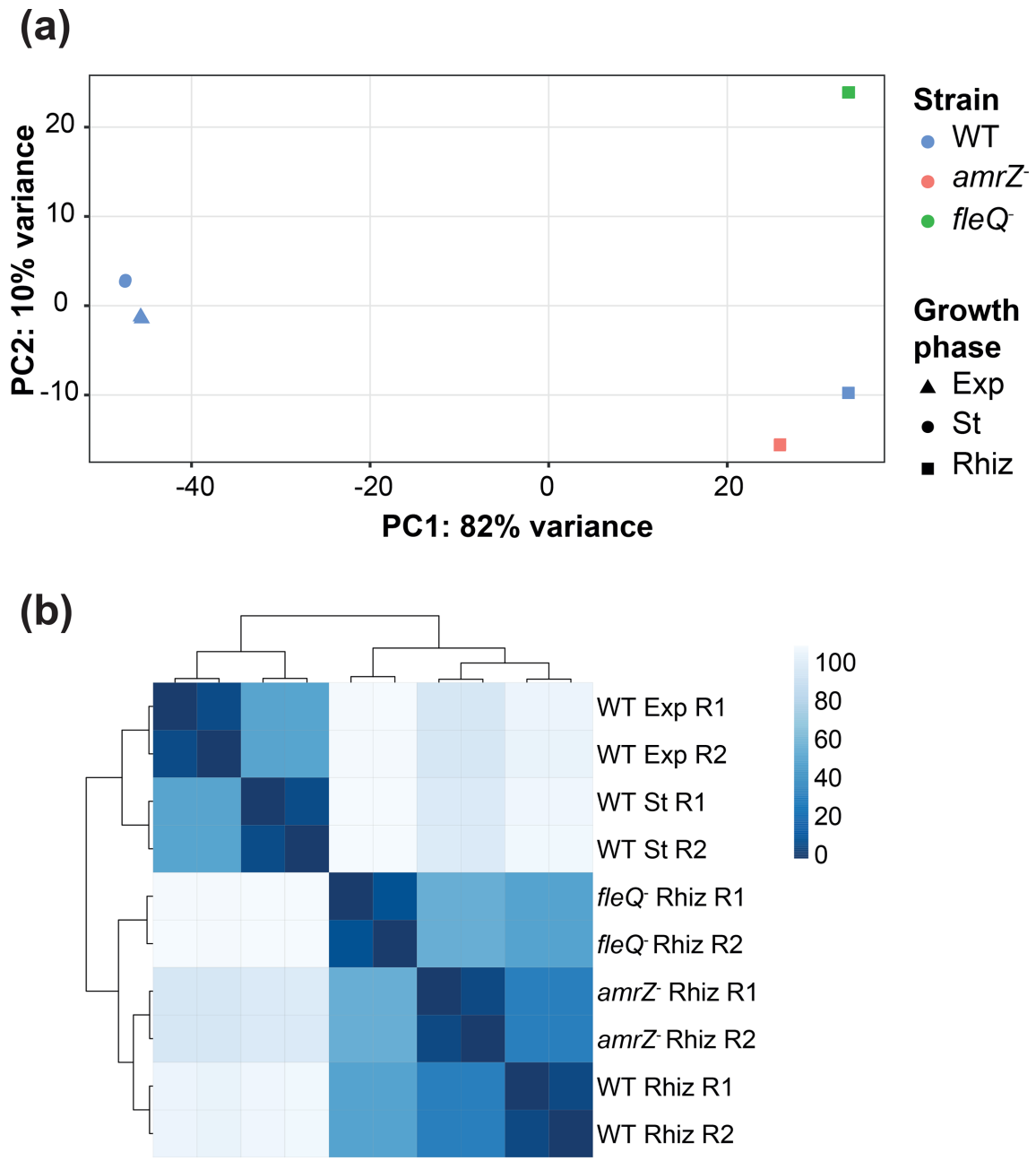

Fig. 1. Gene expression comparison of RNA-Seq samples. Principal Component Analysis (PCA) plot (a) and similarity-based clustering (b) for RNA-Seq samples. Hierarchical clustering is based on Euclidean sample-to-sample distance. WT: wild-type; Exp: Exponential; St: Stationary; Rhiz: Rhizosphere. Different replicates for each sample and condition are represented (R1: replicate 1, R2: replicate 2).

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*

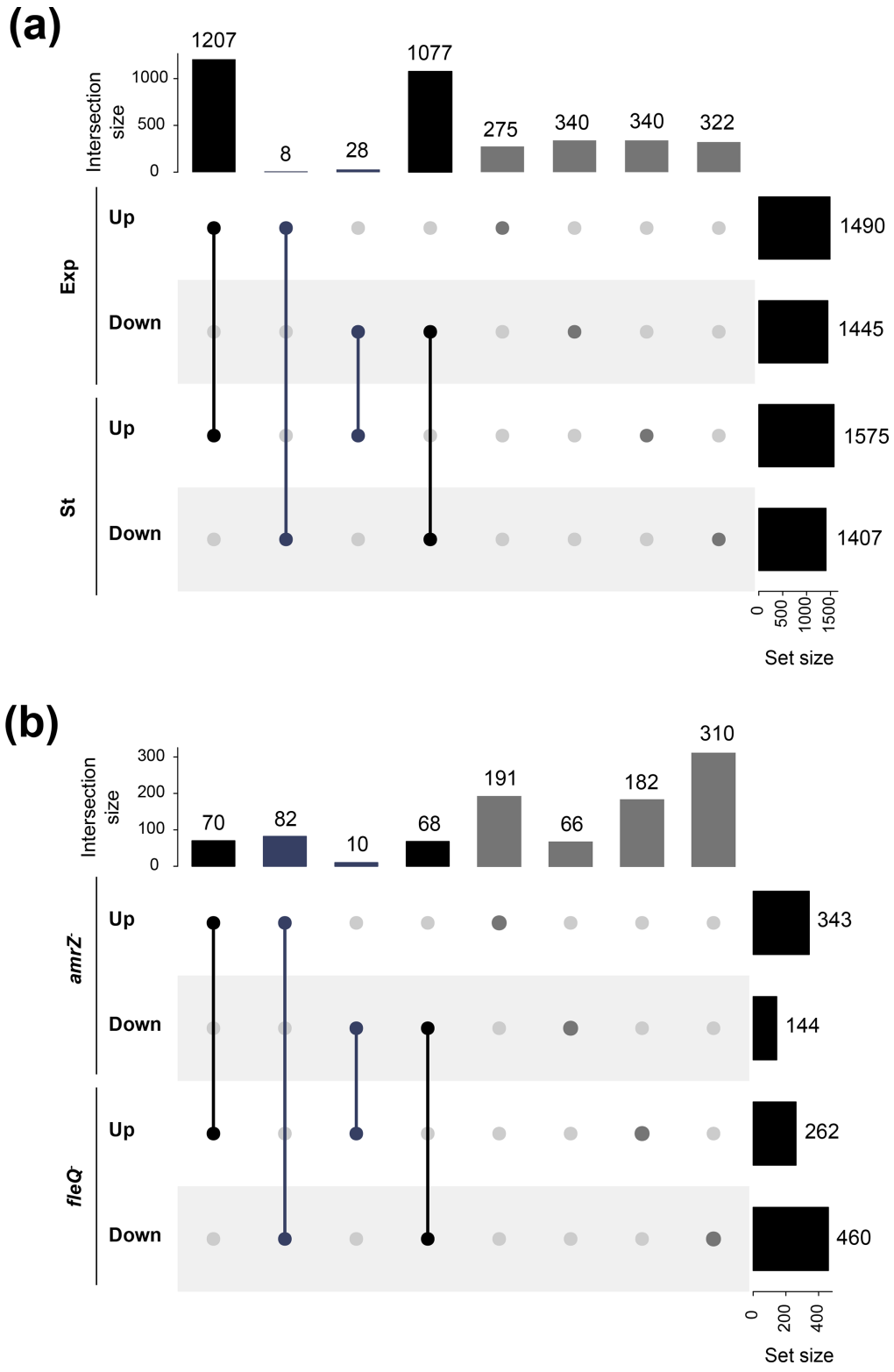

Fig. 2. Differential gene expression in *P. ogarae* F113 and derivatives in different growth conditions. Upset plot showing the intersections of shared and specific up- and down-regulated DEGs among *P. ogarae* F113 grown under culture conditions (a) and the *amrZ* and *fleQ* mutants under rhizosphere conditions (b). DEGs were calculated using F113 wild-type under rhizosphere condition as a reference. DEGs were selected applying the following threshold: $\log_2$ 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. ogarae* 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

Table 1. List of top ten up- and down-regulated genes in *amrZ* and *fleQ* mutants under rhizosphere growth conditions. Locus numbers indicate the 'PSF113_' annotation. Table shows the up- (log_2 Fold-Change (FC) ≥1) and down-regulated genes (log_2 FC ≤−1) with a p-adjusted value ≤0.001. The list with full values is found in File S3. Product is based on the eggNOG database annotations [44].

***amrZ*⁻**			***fleQ*⁻**		
Locus	**Log_2FC**	**Product**	**Locus**	**Log_2FC**	**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, YjgF 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	CspG
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

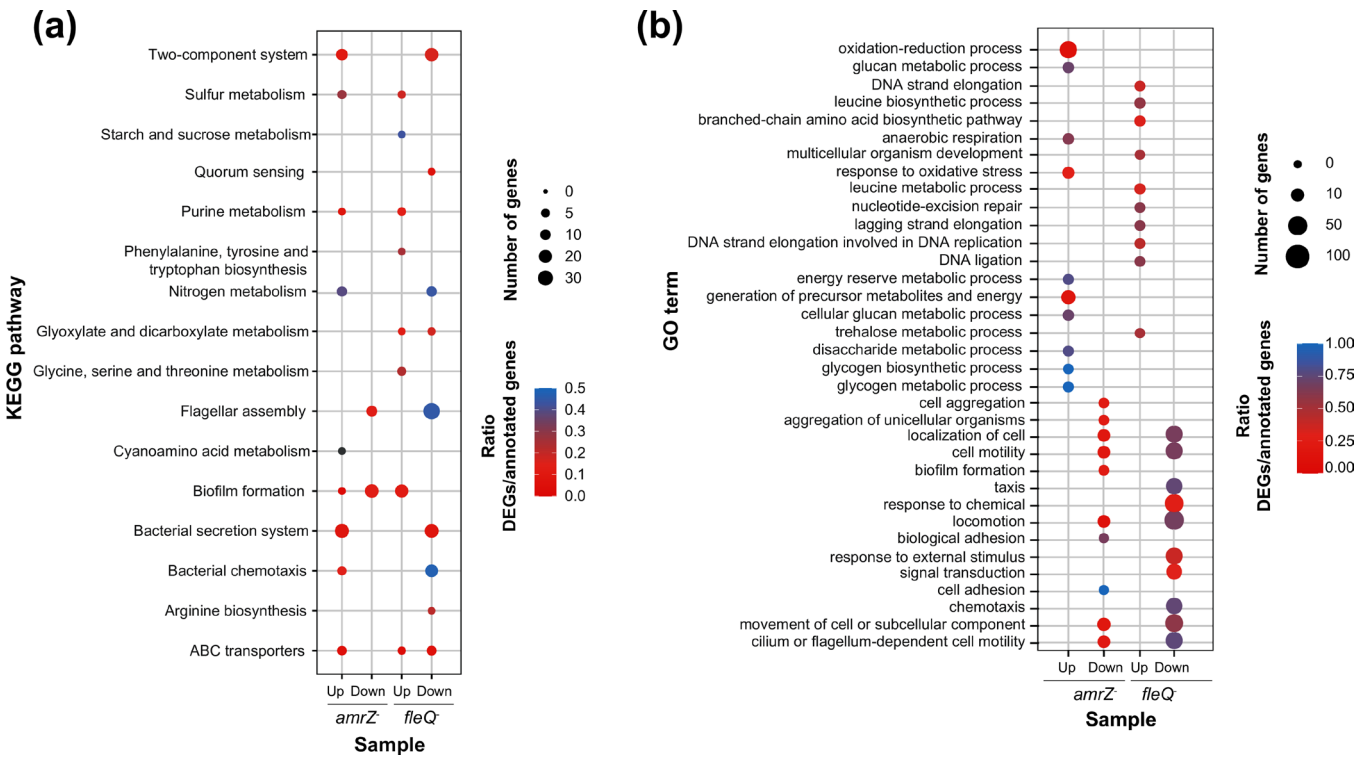

Fig. 3. Functional analysis of differentially expressed genes in *amrZ* and *fleQ* mutants during rhizosphere colonization. Dot plots showing the expression of genes belonging to distinct functional categories according to KEGG (a) and GO (b) databases during rhizosphere growth in the *amrZ* and *fleQ* mutants compared to *P. ogarae* F113 wild-type. Up- and down-regulated KEGG pathways with categories including more than two annotated genes were selected. The dot size is based on the numbers of annotated DEGs found in each case, and the dot colour depicts the ratio of DEGs versus the total of annotated genes in each category (a). Dot plot shows the up- and down-regulated GO terms of Biological processes with a *P*-value < 0.001 in the classic Fisher's test. The dot size shows the number of genes in each term and the colour the ratio between the number of significant genes and the number of annotated genes for each term. The top ten GO terms for each experiment were selected (b).

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. ogarae* 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.

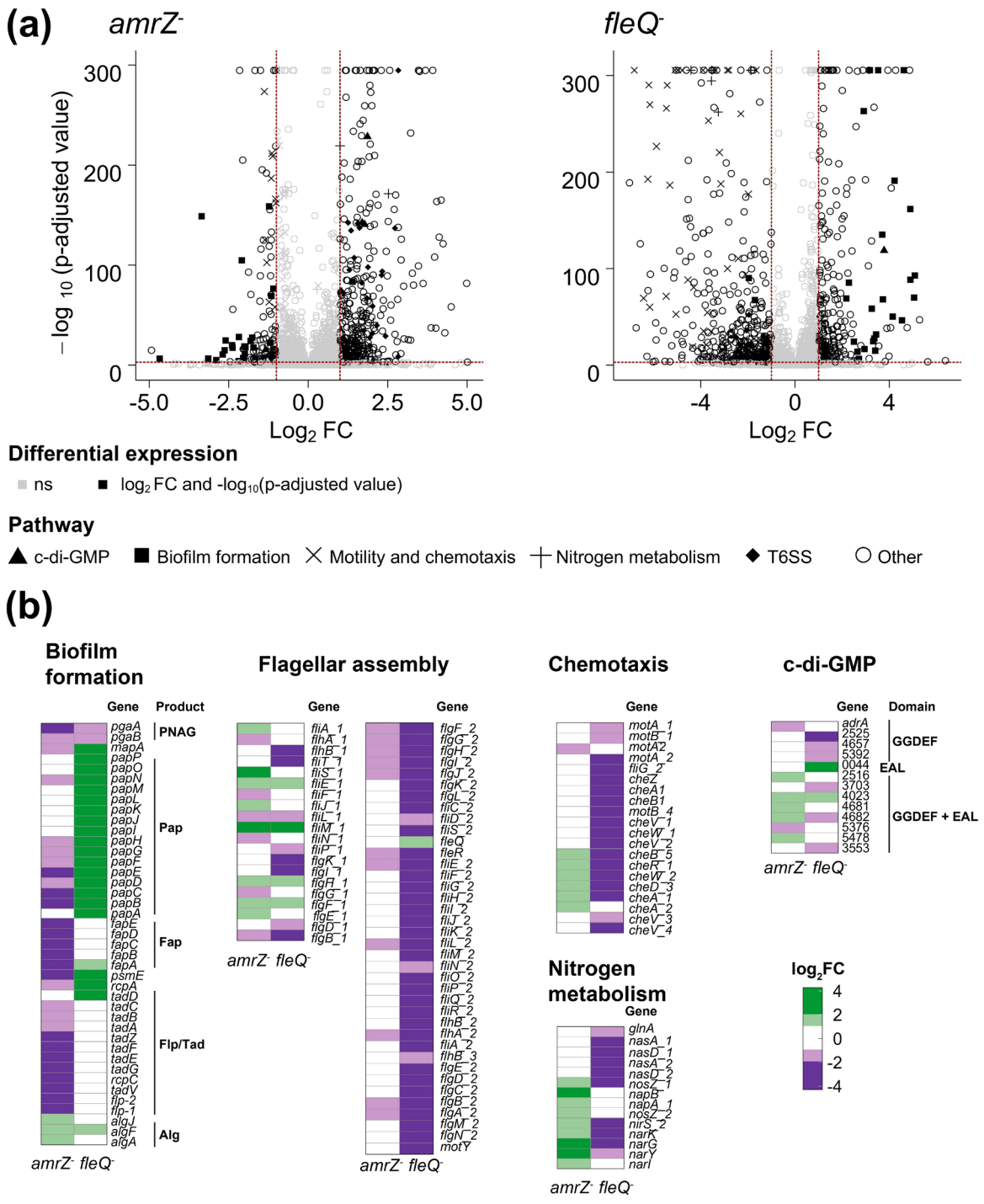

Fig. 4. Differential gene expression of relevant pathways during the rhizosphere colonization process in the *amrZ* and *fleQ* mutants.(a) Volcano plot representing the differential gene expression analysis in the *amrZ* and *fleQ* mutant data sets versus wild-type under rhizosphere conditions. The x-axis shows $\log_2$ Fold-Change (FC) expression and the y-axis the odds of a gene being differentially expressed as the $-\log_{10}$(*P*-adjusted value). Black lined shapes indicate whether genes meet the following threshold: $\log_2$ 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 $\log_2$FC (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, cyanoamino 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 is 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 sulfonate (*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. ogarae* 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. ogarae* 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.

Funding information
This research was funded by Ministerio de Ciencia, Innovación y Universidades FEDER/EU, grant number RTI2018-093991-B-I00. E.B.-R. was granted by FPU-MECD program (FPU16/05513).

Author contributions
Conceptualization: E.B.-R., R.R., M.M. and M.R.-N. *Methodology*: E.B.-R., D.D., D.G.-S. and M.R.-N. *Software*: E.B.-R. and M.R.-N. *Validation*: E.B.-R. and M.R.-N. *Formal analysis*: E.B.-R. and M.R.-N. *Investigation*: E.B.-R. and M.R.-N. *Resources*: E.B.-R. and M.R.-N. *Data curation*: E.B.-R. and M.R.-N. *Writing—original draft preparation*: E.B.-R. *Writing—review and editing*: All authors *Visualization*: E.B.-R., R.R, M.M. and M.R.-N. *Supervision*: R.R., M.M. and M.R.-N. *Project administration*: M.M. and R.R. *Funding*: M.M. and R.R.

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

References
1. Huang H, Shao X, Xie Y, Wang T, Zhang Y, *et al*. An integrated genomic regulatory network of virulence-related transcriptional factors in *Pseudomonas aeruginosa*. *Nat Commun* 2019;10:2931.
2. Pérez-Mendoza D, Felipe A, Ferreiro MD, Sanjuán J, Gallegos MT. AmrZ and FleQ co-regulate cellulose production in *Pseudomonas syringae* pv. tomato DC3000. *Front Microbiol* 2019;10:746.

3. Baynham PJ, Brown AL, Hall LL, Wozniak DJ. *Pseudomonas aeruginosa* AlgZ, a ribbon-helix-helix DNA-binding protein, is essential for alginate synthesis and algD transcriptional activation. *Mol Microbiol* 1999;33:1069–1080.
4. Jones CJ, Newsom D, Kelly B, Irie Y, Jennings LK, *et al*. ChIP-Seq and RNA-Seq reveal an AmrZ-mediated mechanism for cyclic di-GMP synthesis and biofilm development by *Pseudomonas aeruginosa*. *PLoS Pathog* 2014;10:e1003984.
5. Garrett ES, Perlegas D, Wozniak DJ. Negative control of flagellum synthesis in *Pseudomonas aeruginosa* is modulated by the alternative sigma factor AlgT (AlgU). *J Bacteriol* 1999;181:7401–7404.
6. Tart AH, Blanks MJ, Wozniak DJ. The AlgT-dependent transcriptional regulator AmrZ (AlgZ) inhibits flagellum biosynthesis in mucoid, nonmotile *Pseudomonas aeruginosa* cystic fibrosis isolates. *J Bacteriol* 2006;188:6483–6489.
7. Hou L, Debru A, Chen Q, Bao Q, Li K. AmrZ regulates swarming motility through cyclic di-GMP-dependent motility inhibition and controlling pel polysaccharide production in *Pseudomonas aeruginosa* PA14. *Front Microbiol* 2019;10:1847.
8. Waligora EA, Ramsey DM, Pryor EE Jr, Lu H, Hollis T, *et al*. AmrZ beta-sheet residues are essential for DNA binding and transcriptional control of *Pseudomonas aeruginosa* virulence genes. *J Bacteriol* 2010;192:5390–5401.
9. Pryor EE Jr, Waligora EA, Xu B, Dellos-Nolan S, Wozniak DJ, *et al*. The transcription factor AmrZ utilizes multiple DNA binding modes to recognize activator and repressor sequences of *Pseudomonas aeruginosa* virulence genes. *PLoS Pathog* 2012;8:e1002648.
10. Nogales J, Vargas P, Farias GA, Olmedilla A, Sanjuán J, *et al*. FleQ coordinates flagellum-dependent and -independent motilities in *Pseudomonas syringae* pv. tomato DC3000. *Appl Environ Microbiol* 2015;81:7533–7545.
11. Prada-Ramírez HA, Pérez-Mendoza D, Felipe A, Martínez-Granero F, Rivilla R, *et al*. AmrZ regulates cellulose production in *Pseudomonas syringae* pv. tomato DC3000. *Mol Microbiol* 2016;99:960–977.
12. Pérez-Mendoza D, Felipe A, Ferreiro MD, Sanjuán J, Gallegos MT. AmrZ and FleQ co-regulate cellulose production in *Pseudomonas syringae* pv. tomato DC3000. *Front Microbiol* 2019;10:746.
13. Liu H, Yan H, Xiao Y, Nie H, Huang Q, *et al*. The exopolysaccharide gene cluster pea is transcriptionally controlled by RpoS and repressed by AmrZ in *Pseudomonas putida* KT2440. *Microbiol Res* 2019;218:1–11.
14. Baltrus DA, Dougherty K, Diaz B, Murillo R. Evolutionary plasticity of AmrZ regulation in *Pseudomonas*. *mSphere* 2018;3:e00132-18.
15. Huang H, Shao X, Xie Y, Wang T, Zhang Y, *et al*. An integrated genomic regulatory network of virulence-related transcriptional factors in *Pseudomonas aeruginosa*. *Nat Commun* 2019;10:1–13.
16. Arora SK, Ritchings BW, Almira EC, Lory S, Ramphal R. A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. *J Bacteriol* 1997;179:5574–5581.
17. Hu R-M, Yang T-C, Yang S-H, Tseng Y-H. Deduction of upstream sequences of *Xanthomonas campestris* flagellar genes responding to transcription activation by FleQ. *Biochem Biophys Res Commun* 2005;335:1035–1043.
18. Bae N, Park H-J, Park H, Kim M, Do E, *et al*. Elucidating functions of FleQ in *Xanthomonas oryzae* pv. *oryzae* by comparative proteomic and phenotypic analyses. *Int J Mol Sci* 2018;19:10.
19. Hickman JW, Harwood CS. Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol Microbiol* 2008;69:376–389.
20. Wang Y, Li Y, Wang J, Wang X. FleQ regulates both the type VI secretion system and flagella in *Pseudomonas putida*. *Biotechnol Appl Biochem* 2018;65:419–427.
21. Garrido-Sanz D, Meier-Kolthoff JP, Göker M, Martín M, Rivilla R, *et al*. Genomic and genetic diversity within the *Pseudomonas fluorescens* complex. *PLoS One* 2016;11:e0150183.
22. Marshall B, Robleto EA, Wetzler R, Kulle P, Casaz P, *et al*. The adnA transcriptional factor affects persistence and spread of *Pseudomonas fluorescens* under natural field conditions. *Appl Environ Microbiol* 2001;67:852–857.
23. Casaz P, Happel A, Keithan J, Read DL, Strain SR, *et al*. The *Pseudomonas fluorescens* transcription activator AdnA is required for adhesion and motility. *Microbiology (Reading)* 2001;147:355–361.
24. Robleto EA, López-Hernández I, Silby MW, Levy SB. Genetic analysis of the AdnA regulon in *Pseudomonas fluorescens*: nonessential role of flagella in adhesion to sand and biofilm formation. *J Bacteriol* 2003;185:453–460.
25. Giddens SR, Jackson RW, Moon CD, Jacobs MA, Zhang X-X, *et al*. Mutational activation of niche-specific genes provides insight into regulatory networks and bacterial function in a complex environment. *Proc Natl Acad Sci U S A* 2007;104:18247–18252.
26. Garrido-Sanz D, Redondo-Nieto M, Martin M, Rivilla R. Comparative genomics of the *Pseudomonas corrugata* subgroup reveals high species diversity and allows the description of *Pseudomonas ogarae* sp. nov. *Microb Genom* 2021;7.
27. Martínez-Granero F, Redondo-Nieto M, Vesga P, Martín M, Rivilla R. AmrZ is a global transcriptional regulator implicated in iron uptake and environmental adaption in *P. fluorescens* F113. *BMC Genomics* 2014;15:237.
28. Muriel C, Arrebola E, Redondo-Nieto M, Martínez-Granero F, Jalvo B, *et al*. AmrZ is a major determinant of c-di-GMP levels in *Pseudomonas fluorescens* F113. *Sci Rep* 2018;8:1979.
29. Capdevila S, Martínez-Granero FM, Sánchez-Contreras M, Rivilla R, Martín M. Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization. *Microbiology (Reading)* 2004;150:3889–3897.
30. Blanco-Romero E, Redondo-Nieto M, Martínez-Granero F, Garrido-Sanz D, Ramos-González MI, *et al*. Genome-wide analysis of the FleQ direct regulon in *Pseudomonas fluorescens* F113 and Pseudomonas putida KT2440. *Sci Rep* 2018;8:13145.
31. Licciardello G, Ferraro R, Russo M, Strozzi F, Catara AF, *et al*. Transcriptome analysis of *Pseudomonas mediterranea* and *P. corrugata* plant pathogens during accumulation of medium-chain-length PHAs by glycerol bioconversion. *N Biotechnol* 2017;37:39–47.
32. Cornforth DM, Dees JL, Ibberson CB, Huse HK, Mathiesen IH, *et al*. *Pseudomonas aeruginosa* transcriptome during human infection. *Proc Natl Acad Sci U S A* 2018;115:E5125–E5134.
33. Hueso-Gil Á, Calles B, O'Toole GA, de Lorenzo V. Gross transcriptomic analysis of *Pseudomonas putida* for diagnosing environmental shifts. *Microb Biotechnol* 2020;13:263–273.
34. Vesga P, Flury P, Vacheron J, Keel C, Croll D, *et al*. Transcriptome plasticity underlying plant root colonization and insect invasion by *Pseudomonas protegens*. *ISME J* 2020;14:2766–2782.
35. Shanahan P, O'sullivan DJ, Simpson P, Glennon JD, O'gara F. Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl Environ Microbiol* 1992;58:353–358.
36. Martínez-Granero F, Navazo A, Barahona E, Redondo-Nieto M, Rivilla R, *et al*. The Gac-Rsm and SadB signal transduction pathways converge on AlgU to downregulate motility in *Pseudomonas fluorescens*. *PLoS One* 2012;7:e31765.
37. Scher FM, Baker R. Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to fusarium wilt pathogens. *Phytopathology* 1982;72:1567.
38. Durán D, Bernal P, Vazquez-Arias D, Blanco-Romero E, Garrido-Sanz D, *et al*. *Pseudomonas fluorescens* F113 type VI secretion systems mediate bacterial killing and adaption to the rhizosphere microbiome. *Sci Rep* 2021;11:5772.
39. Fahraeus G. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J Gen Microbiol* 1957;16:374–381.
40. Grotzer MA, Patti R, Geoerger B, Eggert A, Chou TT, *et al*. Biological stability of RNA isolated from RNAlater-treated brain tumor and neuroblastoma xenografts. *Med Pediatr Oncol* 2000;34:438–442.

41. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068–2069.
42. Winsor GL, Lam DKW, Fleming L, Lo R, Whiteside MD, *et al*. *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. *Nucleic Acids Res* 2011;39:D596-600.
43. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, *et al*. BLAST+: architecture and applications. *BMC Bioinformatics* 2009;10:1–9.
44. Huerta-Cepas J, Forslund K, Coelho LP, Szklarczyk D, Jensen LJ, *et al*. Fast genome-wide functional annotation through orthology assignment by eggNOG-Mapper. *Mol Biol Evol* 2017;34:2115–2122.
45. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114–2120.
46. Tjaden B. De novo assembly of bacterial transcriptomes from RNA-seq data. *Genome Biol* 2015;16:1.
47. Tjaden B. A computational system for identifying operons based on RNA-seq data. *Methods* 2020;176:62–70.
48. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods* 2017;14:417–419.
49. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:12.
50. Huber W, von Heydebreck A, Sültmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 2002;18 Suppl 1:S96-104.
51. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, *et al*. Gene Ontology: tool for the unification of biology. *Nat Genet* 2000;25:25–29.
52. Kanehisa M. The KEGG database. *Novartis Found Symp* 2002;247:91–101; .
53. Hosack DA, Dennis G, Sherman BT, Lane HC, Lempicki RA. Identifying biological themes within lists of genes with EASE. *Genome Biol* 2003;4:R70.
54. Wickham H. ggplot2. *WIREs Comp Stat* 2011;3:180–185.
55. Kolde R, Kolde MR. Package 'pheatmap. *R Package* 2015;1:790.
56. Conway JR, Lex A, Gehlenborg N. UpSetR: an R package for the visualization of intersecting sets and their properties. *Bioinformatics* 2017;33:2938–2940.
57. Villacieros M, Power B, Sánchez-Contreras M, Lloret J, Oruezabal RI, *et al*. Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (Medicago sativa) rhizosphere. *Plant and Soil* 2003;251:47–54.
58. Perez-Rueda E, Hernandez-Guerrero R, Martinez-Nuñez MA, Armenta-Medina D, Sanchez I, *et al*. Abundance, diversity and domain architecture variability in prokaryotic DNA-binding transcription factors. *PLoS One* 2018;13:e0195332.
59. Sanchez I, Hernandez-Guerrero R, Mendez-Monroy PE, Martinez-Nuñez MA, Ibarra JA, *et al*. Evaluation of the abundance of DNA-Binding transcription factors in prokaryotes. *Genes (Basel)* 2020;11:E52.
60. Blanco-Romero E, Garrido-Sanz D, Rivilla R, Redondo-Nieto M, Martín M. *In silico* characterization and phylogenetic distribution of extracellular matrix components in the model rhizobacteria *Pseudomonas fluorescens* F113 and other pseudomonads. *Microorganisms* 2020;8:11.
61. Wu X, Liu J, Zhang W, Zhang L. Multiple-level regulation of 2,4-diacetylphloroglucinol production by the sigma regulator PsrA in *Pseudomonas fluorescens* 2P24. *PLoS One* 2012;7:11.
62. Matsuyama BY, Krasteva PV, Baraquet C, Harwood CS, Sondermann H, *et al*. Mechanistic insights into c-di-GMP-dependent control of the biofilm regulator FleQ from *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 2016;113:E209-18.
63. Jiménez-Fernández A, López-Sánchez A, Jiménez-Díaz L, Navarrete B, Calero P, *et al*. Complex Interplay between FleQ, Cyclic diguanylate and multiple σ factors coordinately regulates flagellar motility and biofilm development in *Pseudomonas putida*. *PLoS One* 2016;11:e0163142.
64. Baraquet C, Harwood CS. FleQ DNA binding consensus sequence revealed by studies of FleQ-dependent regulation of biofilm gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* 2016;198:178–186.
65. Xiao Y, Nie H, Liu H, Luo X, Chen W, *et al*. C-di-GMP regulates the expression of lapA and bcs operons via FleQ in *Pseudomonas putida* KT2440. *Environ Microbiol Rep* 2016;8:659–666.
66. Nie H, Xiao Y, Liu H, He J, Chen W, *et al*. FleN and FleQ play a synergistic role in regulating lapA and bcs operons in *Pseudomonas putida* KT2440. *Environ Microbiol Rep* 2017;9:571–580.
67. Molina-Henares MA, Ramos-González MI, Daddaoua A, Fernández-Escamilla AM, Espinosa-Urgel M. FleQ of *Pseudomonas putida* KT2440 is a multimeric cyclic diguanylate binding protein that differentially regulates expression of biofilm matrix components. *Res Microbiol* 2017;168:36–45.
68. Xiao Y, Chen H, Nie L, He M, Peng Q, *et al*. Identification of c-di-GMP/FleQ-regulated new target genes, including cyaA, encoding adenylate cyclase, in *Pseudomonas putida*. *mSystems* 2021;6:e00295-21.
69. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2004;2:95–108.
70. Flemming H-C, Wingender J, Szewzyk U, Steinberg P, Rice SA, *et al*. Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* 2016;14:563–575.
71. Barahona E, Navazo A, Yousef-Coronado F, Aguirre de Cárcer D, Martínez-Granero F, *et al*. Efficient rhizosphere colonization by *Pseudomonas fluorescens* f113 mutants unable to form biofilms on abiotic surfaces. *Environ Microbiol* 2010;12:3185–3195.
72. Barahona E, Navazo A, Martínez-Granero F, Zea-Bonilla T, Pérez-Jiménez RM, *et al*. *Pseudomonas fluorescens* F113 mutant with enhanced competitive colonization ability and improved biocontrol activity against fungal root pathogens. *Appl Environ Microbiol* 2011;77:5412–5419.
73. Cole BJ, Feltcher ME, Waters RJ, Wetmore KM, Mucyn TS, *et al*. Genome-wide identification of bacterial plant colonization genes. *PLoS Biol* 2017;15:e2002860.
74. Martínez-Granero F, Rivilla R, Martín M. Rhizosphere selection of highly motile phenotypic variants of *Pseudomonas fluorescens* with enhanced competitive colonization ability. *Appl Environ Microbiol* 2006;72:3429–3434.
75. Muriel C, Jalvo B, Redondo-Nieto M, Rivilla R, Martín M. Chemotactic Motility of *Pseudomonas fluorescens* F113 under aerobic and denitrification conditions. *PLoS One* 2015;10:e0132242.
76. Martínez-García E, Nikel PI, Chavarría M, de Lorenzo V. The metabolic cost of flagellar motion in *Pseudomonas putida* KT2440. *Environ Microbiol* 2014;16:291–303.
77. Barahona E, Navazo A, Garrido-Sanz D, Muriel C, Martínez-Granero F, *et al*. *Pseudomonas fluorescens* F113 can produce a second flagellar apparatus, which is important for plant root colonization. *Front Microbiol* 2016;7:1471.
78. Lucke M, Correa MG, Levy A. The role of secretion systems, effectors, and secondary metabolites of beneficial rhizobacteria in interactions with plants and microbes. *Front Plant Sci* 2020;11:589416.
79. Silverman JM, Brunet YR, Cascales E, Mougous JD. Structure and regulation of the type VI secretion system. *Annu Rev Microbiol* 2012;66:453–472.
80. Allsopp LP, Wood TE, Howard SA, Maggiorelli F, Nolan LM, *et al*. RsmA and AmrZ orchestrate the assembly of all three type VI secretion systems in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 2017;114:7707–7712.
81. Redondo-Nieto M, Barret M, Morrissey J, Germaine K, Martínez-Granero F, *et al*. Genome sequence reveals that *Pseudomonas*

fluorescens F113 possesses a large and diverse array of systems for rhizosphere function and host interaction. *BMC Genomics* 2013;14:54.

82. Taylor TB, Mulley G, Dills AH, Alsohim AS, McGuffin LJ, *et al*. Evolution. Evolutionary resurrection of flagellar motility via rewiring of the nitrogen regulation system. *Science* 2015;347:1014–1017.
83. Sánchez-Contreras M, Martín M, Villacieros M, O'Gara F, Bonilla I, *et al*. Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *J Bacteriol* 2002;184:1587–1596.
84. Köhler T, Harayama S, Ramos JL, Timmis KN. Involvement of *Pseudomonas putida* RpoN sigma factor in regulation of various metabolic functions. *J Bacteriol* 1989;171:4326–4333.
85. Hengge R. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 2009;7:263–273.
86. Little RH, Woodcock SD, Campilongo R, Fung RKY, Heal R, *et al*. Differential regulation of genes for cyclic-di-GMP metabolism orchestrates adaptive changes during rhizosphere colonization by *Pseudomonas fluorescens*. *Front Microbiol* 2019;10:1089.
87. Moe LA. Amino acids in the rhizosphere: from plants to microbes. *Am J Bot* 2013;100:1692–1705.
88. Nelson CE, Huang W, Brewer LK, Nguyen AT, Kane MA, *et al*. Proteomic analysis of the *Pseudomonas aeruginosa* iron starvation response reveals PrrF small regulatory RNA-dependent iron regulation of twitching motility, amino acid metabolism, and zinc homeostasis proteins. *J Bacteriol* 2019;201:12.
89. Hinsa SM, Espinosa-Urgel M, Ramos JL, O'Toole GA. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Mol Microbiol* 2003;49:905–918.
90. Casabona MG, Silverman JM, Sall KM, Boyer F, Couté Y, *et al*. An ABC transporter and an outer membrane lipoprotein participate in posttranslational activation of type VI secretion in *Pseudomonas aeruginosa*. *Environ Microbiol* 2013;15:471–486.
91. Tang R, Zhao L, Xu X, Huang L, Qin Y, *et al*. Dual RNA-Seq uncovers the function of an ABC transporter gene in the host-pathogen interaction between *Epinephelus coioides* and *Pseudomonas plecoglossicida*. *Fish Shellfish Immunol* 2019;92:45–53.
92. Barret M, Morrissey JP, O'Gara F. Functional genomics analysis of plant growth-promoting rhizobacterial traits involved in rhizosphere competence. *Biol Fertil Soils* 2011;47:729–743.
93. Yan Y, Kuramae EE, de Hollander M, Klinkhamer PGL, van Veen JA. Functional traits dominate the diversity-related selection of bacterial communities in the rhizosphere. *ISME J* 2017;11:56–66.