Solvent tolerance mechanisms in Pseudomonas putida

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CHAPTER 6

Characterization of an AraC family transcriptional regulator Afr in *Pseudomonas putida* S12

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Abstract

Megaplasmid curing in *Pseudomonas putida* S12 caused a specific mutation at RPPX_14685 locus, putatively encoding for an AraC family transcriptional regulator (proposed name Afr). Furthermore, during the adaptive laboratory evolution (ALE) experiment to recover solvent tolerance in the plasmid-cured *P. putida* S12, SNPs which cause a frameshift occurred at the *afr* locus. Therefore, Afr seems to play an important role to enable plasmid-curing and regain the solvent tolerance trait in the plasmid-cured strain, however its function remained elusive. In this chapter, we characterize the function and role of Afr. Transcriptional analysis (RNA-seq) and a confirmatory RT-qPCR experiment indicated that Afr positively regulates 32 loci which consist of membrane transporters, porins, and dehydrogenases; including the MexEF-OprN multidrug efflux pump. Remarkably, the mutation and truncation of Afr were able to change the antibiotic resistance profile, underscoring the central role of Afr as a stress-response regulator in *P. putida* S12.

Introduction

The AraC family transcriptional regulators (AFTR) are among the largest families of prokaryotic transcription factors (1). Members of the AFTR typically consist of 250-350 amino acid residues and are ubiquitous in gamma proteobacteria. AFTR proteins contain a variable N-terminal domain and a conserved C-terminal domain, the latter being responsible for DNA binding activity. This regulator family is involved in controlling a wide variety of functions, such as carbon utilization, pathogenesis and stress responses (2). Most AFTRs play a role as positive activators and may respond directly to the environmental signalling molecules which diffused into the bacterial cell or controlled by another regulator (1, 2).

The mechanism of AFTR is modelled after the transcriptional activator AraC of arabinose catabolism gene cluster *ara*BAD. Signalling molecules may interact with the N-terminal effector binding domain (EBD), causing a conformational change of the AFTR dimer and subsequently allows the C-terminal helix-turn-helix (HTH) domain to bind the promoter region (3–5). The proposed mechanism of the AraC transcriptional regulator, called the 'light switch' model, requires the availability of arabinose as an environmental signalling molecule (4). In the absence of arabinose, the AraC dimer will bind the O_2 and I_1 promoter regions. In the presence of arabinose, this dimer undergoes conformational and binding site changes to the I_1 and I_2 promoter regions. This change enables the expression of the *ara*BAD gene cluster and consequently, arabinose utilization; hence the 'light switch' analogy. Other members of AFTRs, such as RegA and ToxT, were also reported to respond to their respective effectors in a similar manner as the 'light switch' model (3, 5). More recently, CmrA was identified to be an AFTR responsible for the antibiotic resistance phenotype of the *nfxC2* mutant *Pseudomonas aeruginosa* PA14 and requires electrophilic molecules to be activated (6).

In previous experiments, we successfully removed the megaplasmid of solvent-tolerant *Pseudomonas putida* S12 and studied its effect on inciting solvent tolerance trait (7). However, the resulting strains carry an identical SNP at RPPX_14685 locus (CDS position 157) which putatively substitutes an amino acid on its N-terminal region. BLASTp searches and protein modelling revealed that RPPX_14685 putatively encodes for an AFTR (proposed gene name *afr*). In this chapter, we characterize the function of this transcriptional regulator and identify the genes regulated by Afr in *P. putida* S12.

Results

A point mutation at the RPPX_14685 locus occurred during plasmid curing with mitomycin C

The megaplasmid pTTS12 was cured from *P. putida* S12 using an intercalating agent, mitomycin C (30 μg ml^{-1}) (7). Following the curing of pTTS12, we performed whole genome sequencing on the plasmid-cured strains to map the mutations that can be caused by plasmid curing using an intercalating agent. The three different plasmid-cured isolates; S12-06, S12-10, and S12-22 contain a limited amount of SNPs (Table 6.1). However, all of the plasmid-cured isolates carry a SNP at the RPPX_14685 locus which resulted in a codon change of ACC into CCC, substituting the 53rd amino acid from threonine into proline (T53P). Upon the repetition of the plasmid curing experiment, all of the plasmid-cured strains were found to contain the same ACC → CCC point mutation (Table 6.2).

Table 6.1. The genetic variants found in the plasmid-cured strains of *P. putida* S12.

Strain	Locus tag	Product	Variant type	Protein effect	Codon change	Amino acid change	CDS pos.	Variant Frequency
S12-06	RPPX_14685	AraC family transcriptional regulator	SNP (transversion)	Substitution	ACC → CCC	T → P	157	100%
S12-10	RPPX_14685	AraC family transcriptional regulator	SNP (transversion)	Substitution	ACC → CCC	T → P	157	100%
S12-22	RPPX_14685	AraC family transcriptional regulator	SNP (transversion)	Substitution	ACC → CCC	T → P	157	100%
S12-22	RPPX_19665	GCN5 family acetyltransferase	Insertion of mobile element	Disruption	-	-	-	-
S12-06	RPPX_05325 - 05330	Intergenic region	SNP (transversion)	-	-	-	-	100%

The recurring ACC → CCC point mutation at the RPPX_14685 locus might give an advantage regarding mitomycin C resistance and might actually be important for losing the

megaplasmid pTTS12. Therefore, we compared the survival rate on mitomycin C and plasmid-curing rate between wild-type S12 and S12c, a S12 strain with the ACC → CCC mutation at RPPX_14685 locus. The strain S12c showed more surviving colonies after mitomycin C treatment, suggesting a higher mitomycin C resistance. All of the surviving colonies of S12c after mitomycin C treatment showed a loss of megaplasmid. In the wild-type S12 strain, only 2.48% of the surviving colonies lost the megaplasmid pTTS12 (Table 6.2). Therefore, the point mutation at the RPPX_14685 locus increased survival and plasmid curing rate during mitomycin C treatment.

Table 6.2. Survival and plasmid curing rate of the wild-type *P. putida* S12 and *P. putida* S12c, carrying ACC → CCC point mutation at the RPPX_14685 locus.

Strain	Survival rate on Mitomycin C (30 μg ml^{-1})	pTTS12 curing rate	Variant frequency at RPPX_14685 locus
S12	2.48 (± 0.58) x 10^{-8}	2.48 (± 0.5) %	100%
S12c	1.44 (±0.51) x 10^{-4}	100%	-

The protein encoded on RPPX_14685 constitutes an AraC family transcriptional regulator (Afr)

Based on our BLASTp search, RPPX_14685 seemed to encode for an **A**raC **f**amily transcriptional **r**egulator and therefore we propose to name this protein Afr. Afr is widely distributed among the *Pseudomonas* genus (Fig 6.1). The best characterized homologue of Afr is CmrA (63% identity, 88% coverage), a transcriptional activator of the MexEF-OprN antibiotic pump found in *Pseudomonas aeruginosa* PA14 (6). *P. putida* S12 also contains another putative AraC family transcriptional regulator encoded on RPPX_17385 locus which shares 58.84% identity and 95% coverage with Afr. This transcriptional regulator is more closely related to CmrA (67% identity, 91% coverage) than Afr, as it belongs to the same clade with CmrA (Fig 6.1). It is interesting to note that other *P. putida* strains (e.g. F1, GB-1, ND6, and DOT-T1E) also contain two Afr-like proteins, one belonging to the same clade as Afr and another to the same clade as CmrA and RPPX_17385.

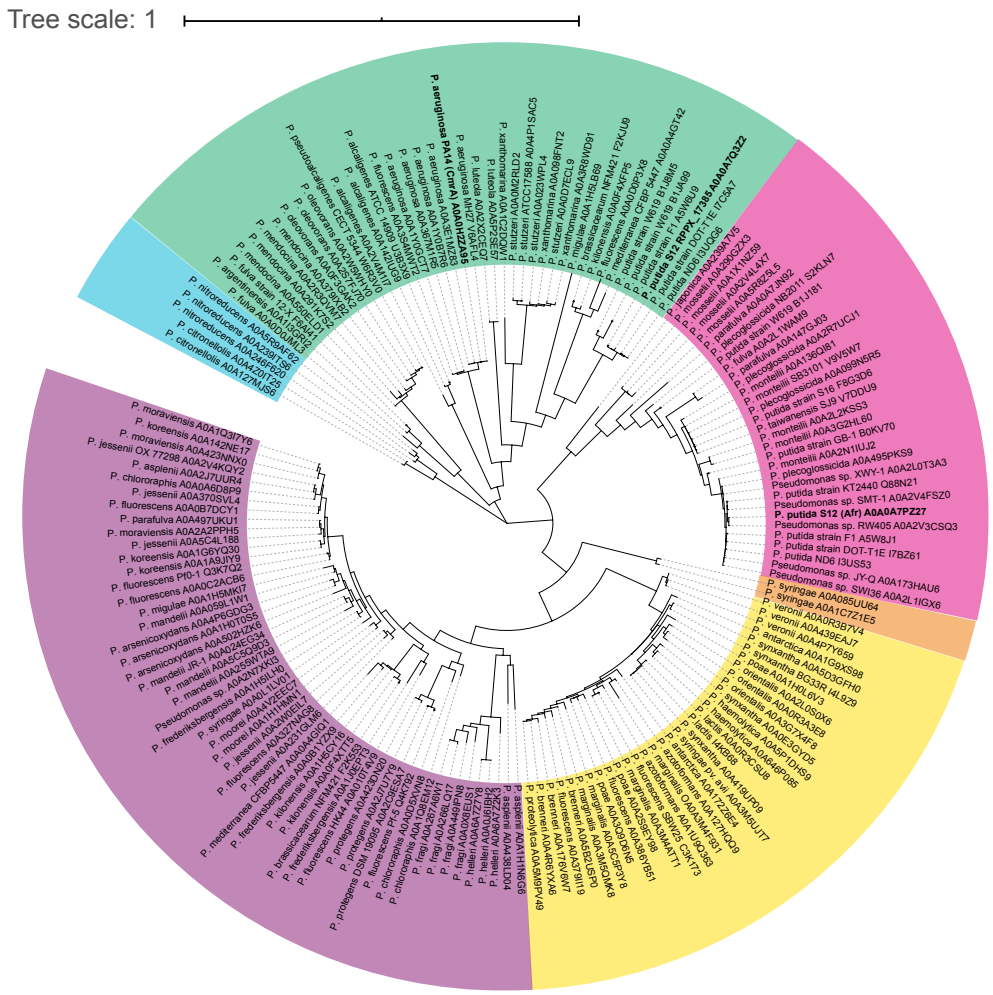

Fig. 6.1. Phylogenetic tree of 165 Afr-like proteins encoded on the genome of various Pseudomonas species. Different colours represent different clades. Afr and CmrA are indicated with bold text, belonging to the magenta and green clade respectively.

Analysis of 165 Afr homologues from different *Pseudomonas* species revealed a high degree of conservation on its C-terminal domain while the N-terminal domain of Afr was highly variable (Fig. 6.2A). The substituted Threonine-53 occurred on this variable N-terminal region (Fig. 6.2A, indicated by the red arrow). It is interesting to note that the HTH-2 C-terminal domain is highly conserved among Afr homologues from different *Pseudomonas* species (Fig. 6.2A). To understand the effect of T53P substitution on Afr, we predicted the structure of Afr using online protein prediction tool I-TASSER (8). Afr was modelled after ToxT from *Vibrio cholerae*, an AFTR which positively regulates the cholera toxin and type VI secretion system

(T6SS), the main characteristics for virulence in *V. cholerae* (5). Afr contains a N-terminal effector binding domain (EBD) and two regions of helix-turn-helix (HTH), which are putatively responsible for DNA binding and the dimerization of the Afr protein (Fig. 6.1B). The substituted Threonine-53 occurred in the middle of the EBD region, at a linker between β-sheets that are largely present on the N-terminal domain (Fig. 6.2B).

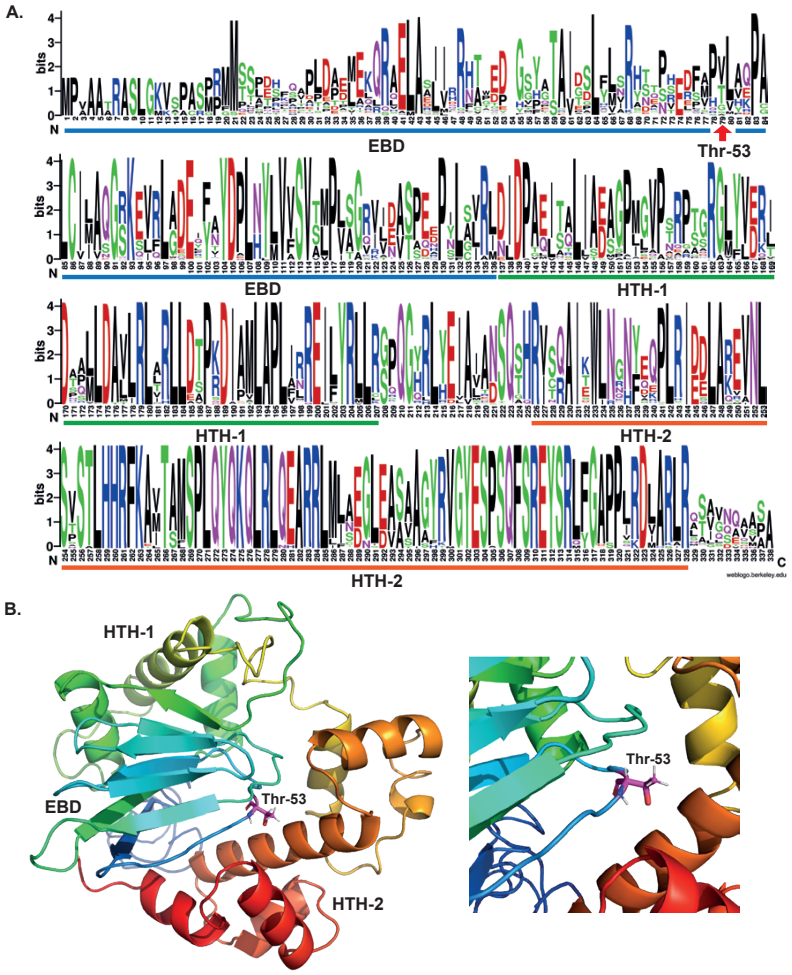

Fig. 6.2. A. Weblogo representation of the amino acid conservation among 165 Afr homologues from different Pseudomonas species.

Different domains of Afr are indicated with colored lines. The substituted Threonine-53 (Thr-53) is indicated with a red arrow. B. I-TASSER prediction of the Afr structure. According to the protein structure of ToxT (PDB ID 3GBG) from Vibrio cholera. Afr contains an effector binding domain (EBD) which consists of parallel beta-sheets and two helix-turn-helix (HTH) regions. The substituted Threonine-53 (Thr-53) is located at the EBD, as indicated in this figure.

Table 6.3. Putative Afr-regulated loci

Locus tag	Annotation	Plasmid-cured S12		Confirmatory RT-qPCR log_2FC; normalized to S12-wt		
		log_2FC	p_value	S12-10	S12-10Δ	S12-10wt
RPPX_14685 *)	AraC family transcriptional regulator	2.20414	5.00E-05	2.448	N/A	-0.006
RPPX_14930	Quinoprotein glucose dehydrogenase	2.07601	5.00E-05	2.377	-0.205	0.078
RPPX_14935	OprB family porin	3.67882	5.00E-05	3.632	0.184	-0.085
RPPX_16940, RPPX_16945	MetNI (methionine importer)	1.32573	5.00E-05	1.590	-0.170	0.336
RPPX_17365	SDR family oxidoreductase	1.72841	5.00E-05	1.700	0.045	0.042
RPPX_17630	DctA (aerobic C4-dicarboxylate transport protein)	5.18095	5.00E-05	3.775	-0.140	-0.197
RPPX_17635	Citrate-divalent cation : H^+ symporter	5.80933	5.00E-05	5.039	-0.087	0.009
RPPX_17640	OprD family porin	3.32728	5.00E-05	3.354	-0.039	0.096
RPPX_17645	CsbD family protein	2.02353	5.00E-05	2.245	0.062	0.082
RPPX_17650	SoxR (bacterial sensor of oxidative stress)	2.06389	5.00E-05	2.076	-0.026	0.024
RPPX_17720	RNA polymerase sigma factor (σ^{70} family)	2.89481	5.00E-05	2.061	-0.012	0.020
RPPX_17725	Diaminopimelate decarboxylase	1.75665	5.00E-05	1.913	0.079	-0.001
RPPX_21210, RPPX_21215 *)	RND efflux pump MexF-OprN	3.9932	5.00E-05	3.772	0.003	-0.043
RPPX_21545	Alginate export family protein	5.14782	5.00E-05	4.432	0.020	-0.005
RPPX_21550	3-(3-hydroxy-phenyl)propionate transporter	2.35045	5.00E-05	2.046	-0.089	-0.131
RPPX_21555	RbdA	3.50752	5.00E-05	3.778	-0.178	0.237
RPPX_21560, RPPX_21565	TonB-dependent receptor	3.61084	5.00E-05	3.515	0.032	-0.045
RPPX_24075	Zinc-dependent alcohol dehydrogenase	2.2774	5.00E-05	2.225	0.115	0.012
RPPX_24520	Quinoprotein dehydrogenase	2.24277	5.00E-05	2.227	0.091	0.008
RPPX_24525	Arginine binding protein	1.82785	5.00E-05	1.737	-0.013	0.249
RPPX_24930, RPPX_24935 *)	IorAB (Isoquinoline 1-oxidoreductase)	5.37876	5.00E-05	4.836	-0.278	0.011
RPPX_25035	Methyl-accepting chemotaxis protein	3.56493	5.00E-05	3.704	-0.012	0.057
RPPX_25340	Enoyl-CoA hydratase	5.01552	5.00E-05	4.937	-0.080	0.073
RPPX_25345, RPPX_25350, RPPX_25355	Putative ABC transport system	1.73293	5.00E-05	1.912	0.140	0.227

**) homologs of these genes were also regulated by CmrA from PA14 (6)*

***) Colours indicate putative gene clusters*

Identification of the genes regulated by Afr

Previous RNA-seq experimental data were analysed to identify the genes regulated by Afr. In this experiment, we compare the wild-type S12 with the plasmid-cured S12, the latter contains the T53P substitution at Afr locus (see Chapter 5). The RNA-seq data were screened for the genes that are differentially expressed in the plasmid-cured S12 compared to the wild-type S12. We found 32 loci differentially expressed in this comparison and all of these loci were upregulated in the plasmid-cured S12 compared to the wild-type S12 (Table 6.3).

We performed RT-qPCR to confirm our findings of the genes putatively regulated by Afr. For this experiment, we used the wild-type S12 as the control strain. Strain S12-10 represented the plasmid-cured strains, containing only the point mutation ACC → CCC at *afr* locus. Strain S12-10wt represented the plasmid-cured strain without the point mutation at the *afr* locus (wild-type Afr). Strain S12-10Δ represented the strain with a deleted *afr* locus in the absence of pTTS12 plasmid. The 32 loci were upregulated in the strain S12-10 while there were no observed differences in the expression of these loci in the strains S12-10wt and S12-10Δ compared to the wild-type S12. Indeed, all of the 32 loci that are putatively regulated by Afr according to the RNA-seq data were confirmed to be regulated by Afr in our RT-qPCR experiment.

Many of these loci appear to constitute putative gene clusters, indicated by different colours on Table 6.3. Moreover, several loci belong to polycistronic mRNA, for instance, RPPX_16940-RPPX_16945, RPPX_21210-21215, RPPX_24930-RPPX_24935, and RPPX_25345-RPPX_25355. Among the 32 loci regulated by Afr, the genes encoded by RPPX_14685 (Afr), RPPX_21210 & RPPX_21215 (MexF-OprN), and RPPX_24930 & RPPX_24935 (aldehyde dehydrogenase & oxidoreductase) are homologs to the genes regulated by CmrA from *P. aeruginosa* PA14 (6), as indicated by asterisks on Table 6.3.

The T53P substitution of Afr affected antibiotic resistance profile of *P. putida* S12

Based on the RNA-seq and RT-qPCR findings, the substitution T53P at the Afr locus (strain S12-10) seemed to cause an upregulation of RPPX_21210 and RPPX_21215 which encode for MexF and OprN, the inner and outer membrane components of the MexEF-OprN efflux pump respectively. Therefore, the point mutation at the *afr* locus may change the antibiotic resistance of the plasmid-cured S12 strain. Indeed, we demonstrated an increase in the survival rate of the strain S12c during mitomycin C treatment (Table 6.2). To characterize the effect of

the T53P substitution in Afr, we quantified the minimum inhibitory concentration (MIC assay) of several antibiotics on the strains S12, S12-10, S12-10wt, and S12-10Δ (Table 6.4).

The strain S12-10 showed an increase in its antibiotic resistance against Mitomycin C (8 times), ciprofloxacin (8 times) and chloramphenicol (4 times) compared to the wild-type S12 strain (Table 6.4). Ciprofloxacin and chloramphenicol have been previously identified as substrates of the MexEF-OprN pump (6). The strains S12-10wt and S12-10Δ showed a similar antibiotic resistance profile as the wild-type S12 strain, which confirms the RNA-seq and RT-qPCR findings that in these strains the expression level of the MexEF-OprN efflux pump was similar to the wild-type S12. This indicates that in the strain S12-10, the MexEF-OprN was indeed upregulated. However, a decrease in antibiotic resistance against carbenicillin, kanamycin, and streptomycin was observed in the strain S12-10. These antibiotics are not the substrates of MexEF-OprN; they are the substrates of ArpABC, a homolog to TtgABC from *P. putida* DOT-T1E and MexAB-OprM from *P. aeruginosa* (9, 10).

Table 6.4. Minimum inhibitory concentration of several antibiotics in *P. putida* S12 strains

Antibiotic	Minimum inhibitory concentration (μg ml^{-1})			
	S12-wt	S12-10wt	S12-10	S12-10Δ
Mitomycin C	32	32	256	32
Carbenicillin	256	256	64	256
Cefotaxime	16	16	16	16
Kanamycin	32	32	16	32
Ciprofloxacin	0.125	0.125	1	0.125
Trimethoprim	4096	4096	4096	4096
Aztreonam	32	32	16	32
Imipenem	2	2	2	2
Chloramphenicol	128	128	512	128
Streptomycin	64	64	32	64
Tetracycline	16	16	16	16

Discussion

The substitution T53P putatively caused a constitutive activation of Afr

Similar to CmrA (6) and ToxT (5), Afr seemed to constitute a positive regulator in the family of

AraC transcriptional regulators. This family of transcriptional regulators requires an induction, e.g. Arabinose for the model AraC transcriptional regulator in *E. coli* (11) and cinnamaldehyde for CmrA in *P. aeruginosa* PA14 (6). In our findings, S12-10wt and S12-10Δ did not seem to show a differential expression on the 32 loci putatively regulated by Afr relative to the wild-type S12 strain. Indeed, this may indicate that in the wild-type S12 and S12-10wt, Afr is present in an inactive form. The substitution T53P occurred at the effector binding domain (EBD) region of Afr. The EBD region is important for the binding to the inducer and thus drives the activity of an AraC family transcriptional regulator (AFTR) to form a dimer and bind to the promoter regions (5, 11). Our data indicated that the T53P substitution caused Afr to be in a constitutively active form, overriding the need for an inducer, and therefore causing a constitutive upregulation of its target loci.

BLASTp analysis revealed that the Afr protein is ubiquitous in *Pseudomonas* species. Indeed, several *Pseudomonas putida* strains even contain two Afr proteins, e.g. RPPX_14685 and RPPX_17385 from *P. putida* S12. The C-terminal region composing an HTH-2 domain exhibits a high amino acid sequence conservation. This region is responsible for DNA binding, while the effector binding domain (EBD) and the HTH-1 domain is responsible for the dimerization of Afr-like protein (3, 5, 12). Due to the high conservation of the HTH-2 domain and the low conservation of EBD, it is possible that the two Afr-like proteins in *P. putida* strains recognize the same promoter regions and regulate the same set of genes while responding to different environmental effectors. It is interesting to study the cross-recognition among similar AFTRs which exist in one strain.

Afr regulates a plethora of membrane-bound proteins

Several Afr-regulated genes share homology with the genes regulated by CmrA from *P. aeruginosa* PA14. RPPX_21210 and RPPX_21215, which encode for MexF and OprN respectively, are among the Afr-regulated loci. In the strain S12-10 with T53P substitution on Afr, we could observe an increase of the minimum inhibitory concentration (MIC) of MexEF-OprN substrates like ciprofloxacin and chloramphenicol. Afr seemed to be self-regulating similar to its homolog CmrA in *P. aeruginosa* PA14 (6). In addition, Afr and CmrA also seem to regulate a putative membrane-bound isoquinoline-1-oxidoreductase (*iorAB*) encoded on RPPX_24930 and RPPX_24935 in *P. putida* S12 or PA1881 and PA1880 in *P. aeruginosa* PA14. This reductase was characterized for its ability to catalyse the hydroxylation of isoquinoline to

1-oxo-1,2-dihydroxyquinoline (13). A recent study also identified this reductase to be responsible for vanillin utilization in *P. putida* KT2440 as an alternative vanillin dehydrogenase (14).

Our RNA-seq and RT-qPCR data suggested that Afr regulates other membrane proteins like porins and transporters that are responsible for the transport of carbon sources and amino acids. RPPX_14635 and RPPX_17640 are predicted to encode for a carbohydrate-specific OprB porin and an amino acid porin OprD, respectively (15, 16). RPPX_16940 and RPPX_16945 are predicted to encode for the homolog of MetNI, a high-affinity methionine ABC transporter previously described in *E. coli* (17). Furthermore, RPPX_17630 and RPPX_17635 appear to encode for proton-dependent C4-dicarboxylate and citrate-Mg^{2+}/Ca^{2+} symporters, respectively (18). We attempted to predict the DNA binding sites of Afr using MEMEsuite (http://meme-suite.org/) based on the upstream regions of the Afr-regulated gene clusters. However, we could not identify any putative binding site from this analysis. Indeed, Afr may not directly regulate the expression of these transporters and membrane proteins. Further binding site analysis (e.g. CHIP-seq) may be beneficial to elucidate the regulation mechanism of Afr.

The role of Afr in enabling plasmid-curing and restoring solvent tolerance in ALE-derived strains

Previous attempts reported success in curing *Pseudomonas* plasmids using Mitomycin C treatment with a plasmid curing rate of 5-7% (19, 20). In the plasmid-cured strains, a point mutation occurred at *afr* locus causing the S12c strain to have a higher tolerance towards mitomycin C and subsequently, a higher plasmid-curing efficiency. This mutation seemed to be strongly selected for by mitomycin C treatment, possibly caused by its role in increasing tolerance towards mitomycin C. Upregulation of the MexEF-OprN antibiotic pump regulated by Afr caused the increase of surviving colonies after mitomycin C treatment.

A frameshift at the *afr* locus is among the key mutations required for the restored tolerance towards high toluene concentrations in ALE-derived strains (Chapter 5). Truncation of the Afr protein and downregulation of the loci it is regulating, are therefore essential for the restoration of the solvent tolerance trait. Afr regulates an antibiotic efflux pump and several transporters which require H^+ influx to energize these systems, thus competing with the ArpABC efflux pump for toluene extrusion. Moreover, Afr positively regulates several porins which can be the entry channel for organic solvent into cytoplasm and membrane lipid bilay-

er. While the wild-type Afr requires an induction for upregulating these loci, the mutated Afr (T53P) is constitutively active and therefore these loci are constitutively overexpressed. The frameshift at the *afr* locus in ALE derived strains probably is required to maintain proton motive force and reduce toluene influx through porins.

Interplay between MexEF-OprN and ArpABC is mediated by Afr

In our plasmid-cured strains, the substitution T53P at Afr caused an increase in the MIC value of chloramphenicol and ciprofloxacin, the known substrates of MexEF-OprN. However, the MIC values for carbenicillin, kanamycin and streptomycin, which are the substrates of ArpABC, were decreased even though the expression level of ArpABC was not affected. In the ALE-derived strains, the ArpABC efflux pump was upregulated while other RND efflux pumps and proton-dependent membrane transporters were downregulated, including the MexEF-OprN. A previous study in *P. aeruginosa nfxC* mutant having the overexpression of MexEF-OprN pump, described a decreased production of C4-HSL which causes the downregulation of MexAB-OprM pump (homolog to ArpABC) (10). Taken together, there seems to be a competition between the efflux pumps ArpABC and the MexEF-OprN mediated by genetic regulatory elements like Afr and C4-HSL.

A previous study indicated that the expression of multiple pumps can cause a deleterious effect on the strain harbouring them (21). The cause of this toxicity may be due to the maintenance of proton motive force or the spatial competition since these pumps are embedded on the limited surface of the cell membrane. Afr seems to have a central role in the interplay between ArpABC and MexEF-OprN by positively regulating the expression of MexEF-OprN. Differential expression of MexEF-OprN, and perhaps also other membrane-bound transporters, can affect the performance of ArpABC in conferring antibiotic resistance or solvent tolerance to *P. putida* S12.

Materials and Methods

Strains and culture conditions

Strains and plasmids used in this chapter are listed in Table 6.5. All *P. putida* strains were grown in Lysogeny Broth (LB) at 30 °C with 200 rpm shaking and *E. coli* strains were cultivated in LB at 37 °C. For solid cultivation, 1.5 % (wt/vol) agar was added to LB. When required, gentamicin (25 mg liter^{-1}), carbenicillin (32 - 4096 mg liter^{-1}), kanamycin (1 - 128 mg liter^{-1}),

cefotaxime (2 – 256 mg liter^{-1}), ciprofloxacin (0.0625 – 8 mg liter^{-1}), trimethoprim (0.128 – 16.384 g liter^{-1}), aztreonam (2 - 256 mg liter^{-1} in DMSO), chloramphenicol (32 - 4096 mg liter^{-1} in methanol), streptomycin (8 – 1024 mg liter^{-1}), tetracycline (0.5 – 64 mg liter^{-1}), were added to the media.

Table 6.5. Strains and plasmids used in this chapter

Strain	**Characteristics**	**Ref.**
P. putida S12	Wild-type *P. putida* S12 (ATCC 700801), harboring megaplasmid pTTS12	(22)
P. putida S12c	*P. putida* S12, contains the point mutation ACC → CCC at RPPX_14685 locus (CDS. position 157)	(7)
P. putida S12-10	*P. putida* S12 ΔpTTS12, contains the point mutation ACC → CCC at RPPX_14685 locus (CDS. position 157)	(7)
P. putida S12-10wt	*P. putida* S12 ΔpTTS12, contains the wild-type RPPX_14685 locus (CDS. position 157)	This paper
P. putida S12-10Δ	*P. putida* S12 ΔpTTS12 ΔRPPX_14685	This paper
E. coli WM3064	*thrB1004 pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)567 Δ*dapA1341*::[erm pir]	William Metcalf
Plasmid	**Descriptions**	**Ref.**
pEMG	Km^R, Ap^R, *ori* R6K, *lacZ*α MCS flanked by two I-SceI sites	(23)
pEMG-Δ*afr*	pEMG plasmid for constructing *P. putida* S12-10Δ	This paper
pEMG-*afr*_wt	pEMG plasmid for constructing *P. putida* S12-10wt	This paper
pSW-2	Gm^R, *ori* RK2, *xylS*, Pm → I-SceI	(23)

DNA and RNA methods

All PCRs were performed using Phusion polymerase (Thermo Fisher) according to the manufacturer's manual. Primers used in this chapter (Table 6.6) were procured from Sigma-Aldrich. PCR products were visualized by gel electrophoresis on 1 % (w/v) TBE agarose containing 5 µg ml^{-1} ethidium bromide (110V, 0.5x TBE running buffer). For whole genome sequencing, DNA was extracted and the obtained data was processed as described in Chapter 5. The genome sequence of the plasmid-cured *P. putida* S12 have been submitted to the SRA database under accession number PRJNA602416.

For RT-qPCR analysis, RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's manual. The obtained RNA samples were cleaned-up using NucleoSpin® RNA Plant and Fungi kit (Macherey-Nagel) and immediately reverse transcribed using iScript™ cDNA synthesis kit (BioRad). cDNA may be stored at -20 °C prior to qPCR analysis. qPCR was performed using iTaq™ Universal SYBR Green Supermix (BioRad) on CFX96 Touch™ Real-Time PCR Detection System (BioRad). Data analysis was performed to calculate the $\log_2$ fold-change value as described by Taylor and colleagues (24), using the wild-type S12 strain as the control strain where *rpoB* and *gyrB* were chosenas the control household genes. For RNA-seq, the experiment was carried out as described in Chapter 5. Datasets generated from the RNA-seq experiment have been submitted to the GEO database under accession number GSE144045.

Table 6.6. Oligos used in this study

Oligos	Sequence	Purpose
KO_TS1_Afr_F	CCTCAGGATCCCTCAGTCGAAGGTATGCAAGTC-CA	knocking-out RPPX_14685
KO_TS1_Afr_R	TTCTTCACCTTTATGAATTCCCATGATCATGATG-GCTTTCCTCTGCTGACCG	knocking-out RPPX_14685
KO_TS2_Afr_F	CGCACGGCATGGATGAACTCTACAAATAAAGCT-CAACATTCGCCTGCACCG	knocking-out RPPX_14685
KO_TS2_Afr_R	GGCGATCTAGACCAACCTGTGTATACCGG-CAACCT	knocking-out RPPX_14685
c_Afr_F	GCAATGAATTCATCTGTGCAAAAACCTGTA	complementation of RPPX_14685 to the wild-type sequence in S12-10wt strain
c_Afr_R	AGGGTTCTAGAACAATCTCCTTGAAGCAG	complementation of RPPX_14685 to the wild-type sequence in S12-10wt strain
eco_gyrB_F	CGATAATTTTGCCAACCACGAT	qPCR, reference gene
eco_gyrB_R	GAAATTCTCCTCCCAGACCAAA	qPCR, reference gene
eco_rpoB_F	AACACGAGTTCGAGAAGAAACT	qPCR, reference gene
eco_rpoB_R	CGTTTAACCGCCAGATATACCT	qPCR, reference gene
ppu_gyrB_F	GCTTCGACAAGATGATTTCGTC	qPCR, reference gene
ppu_gyrB_R	GCAGTTTGTCGATGTTGTACTC	qPCR, reference gene
ppu_rpoB_F	GACAAGGAATCGTCGAACAAAG	qPCR, reference gene
ppu_rpoB_R	GAAGGTACCGTTCTCAGTCATC	qPCR, reference gene
14685_F	GCAGTTGCTTCTGGTACTGA	qPCR, target gene

Oligos	Sequence	Purpose
14685_R	GCTCAACAACAACTTCCAAC	qPCR, target gene
14930_F	GATGATGTAATCGCCCATCT	qPCR, target gene
14930_R	CAAGGAGTTGTGGAAAGCTC	qPCR, target gene
14935_F	CAAGGGAAGCTGTTGAAGTC	qPCR, target gene
14935_R	GATGTGGATCAAGCAGAAGTAC	qPCR, target gene
16940_F	CTGATCCTGGACGAACCTAC	qPCR, target gene
16940_R	GTTCAGCCTCTTCCAGGTAGT	qPCR, target gene
16945_F	GATCACCATGGTCCTGTACTT	qPCR, target gene
16945_R	ATGTACTGCATGTAGGTGAAGC	qPCR, target gene
17365_F	TGCAGCAATACCGTGTAGTAC	qPCR, target gene
17365_R	GTCGACGTACTCATCAACAATG	qPCR, target gene
17630_F	GAGAAACTCGAGAAGCTTGG	qPCR, target gene
17630_R	AACGACAGATAGATGGCAGTAC	qPCR, target gene
17635_F	CACCACGACTTACATGATTACC	qPCR, target gene
17635_R	CTCATGATGCTCAATGACAG	qPCR, target gene
17640_F	CAACGTGTACTTCAACGAGA	qPCR, target gene
17640_R	GTAATCGAGCAGAAAGCCTT	qPCR, target gene
17645_F	GTAGGTCTTGGTCACCTGG	qPCR, target gene
17645_R	ATCGAAGGTGTAGCAGAAGAC	qPCR, target gene
17650_F	CTGGGCTACCTTGATGATC	qPCR, target gene
17650_R	CTGCACTTCTACGAAACCAAG	qPCR, target gene
17720_F	CCACCAGCATTTCTATGATG	qPCR, target gene
17720_R	CCTTCTGATAGACAAAGCTCG	qPCR, target gene
17725_F	CTGTTGTAGTTCGACGACATC	qPCR, target gene
17725_R	GTGTTCACTCAGGATGACCA	qPCR, target gene
21210_F	AGTACCAGGTAGTCCACGGTAC	qPCR, target gene
21210_R	TCAGCGATTACGACAAGAC	qPCR, target gene
21215_F	GTGTACAGTGAACCCAGGTAAAC	qPCR, target gene
21215_R	CTGTTTACCAGCTACCAGGT	qPCR, target gene
21220_F	TGTCGACGAAGTTCATCTG	qPCR, target gene
21220_R	GTACCTCAAGTACACCCAGCT	qPCR, target gene
21545_F	CTGAACGACGATTCCTATCT	qPCR, target gene
21545_R	GAGTACAAGTCCTTGCCAAAG	qPCR, target gene
21550_F	GTGTGCTTACCCAACTGTTC	qPCR, target gene
21550_R	GCAACATGTACAACACTGTCAG	qPCR, target gene
21555_F	GAACGTGTCGTTGATCTGTT	qPCR, target gene
21555_R	AACTGACTGGTCTGAACAACC	qPCR, target gene
21560_F	CTTCCAGGGTGAATAGACAAC	qPCR, target gene
21560_R	GACTTCACCAACGAGATGAC	qPCR, target gene

Oligos	Sequence	Purpose
21565_F	GTAGTGGTAGAAGGTATTACGCAG	qPCR, target gene
21565_R	CACTCTGCACATCGACAAG	qPCR, target gene
24075_F	GGTACACCTTGATGATCTGC	qPCR, target gene
24075_R	CAGTTCTATGTGCACTGCATC	qPCR, target gene
24520_F	GTCGATCTCCAGCGGTAC	qPCR, target gene
24520_R	CTTCTGTGATGTGGGACTTCTAC	qPCR, target gene
24525_F	GTCATCGTCGAGCTTTTCTC	qPCR, target gene
24525_R	CCTACAGTTTCCAGGACCAC	qPCR, target gene
24930_F	CATCAACCAGAAGACCTATCAG	qPCR, target gene
24930_R	GCAGCCATACTTGGTGC	qPCR, target gene
24935_F	ATGCAGGTAGACTGGAAAGAAC	qPCR, target gene
24935_R	ATATCCCCTTCGTTCTCTTC	qPCR, target gene
25035_F	AACTGCACACTGTCACTGAAG	qPCR, target gene
24035_R	GCAGTCATCTGGTTCACC	qPCR, target gene
25340_F	GAACGACCTGATCACACG	qPCR, target gene
25340_R	GTACATGGCAGTGTTCAGG	qPCR, target gene
25345_F	CTGTAATAGTACGAAGCCTGG	qPCR, target gene
25345_R	GTATTGAACAGCTACAGATGGG	qPCR, target gene
25350_F	GAACACATAGAGCGGTAACC	qPCR, target gene
25350_R	CAGACACTCATGCTCAGCA	qPCR, target gene
25355_F	GATCAGGTTGTACTGCTGAAAC	qPCR, target gene
25355_R	GTATTCTGATCGACGACGTTC	qPCR, target gene

Curing and complementation of megaplasmid pTTS12 from *P. putida* S12

P. putida S12 was grown in LB to reach early exponential phase (± 3 hours or OD_{600} = 0.4-0.6). Subsequently, mitomycin C was added to the liquid culture to a final concentration of 10, 20, 30, 40, or 50 μg ml^{-1}. These cultures were grown for 24 hours and plated on M9 minimal media plated supplemented with indole to select for the absence of the megaplasmid. Complementation of the megaplasmid pTTS12 was performed using bi-parental mating between *P. putida* S12 (pTTS12 Km^R) and plasmid-cured strain *P. putida* S12 ΔpTTS12 (Gm^R :: Tn7) and followed by selection on LB agar supplemented with kanamycin and gentamicin.

MIC assay

The quantification of minimum inhibitory concentration (MIC) values were performed using broth microdilution on a 96-well plate. Overnight starting culture was diluted with LB media to reach 0.5 McFarland or approximately 10^8 CFUs ml^{-1}. A mixture of 95 µl LB media and 5 µl of

the inoculum suspension was placed in each well of a 96-well plate. Each column represented a different sample to be analyzed, with column 1-3 representing the wild-type S12, column 4-6 representing strain S12-10wt, column 7-9 representing strain S12-10, and column 10-12 representing strain S12-10Δ. Antibiotic solution (100 µl, diluted from the stock solution accordingly) was placed in row A and thoroughly mixed by pipetting. From the first row of wells, 100 µl of the mixture was added to the second row and mixed, and this serial dilution procedure was repeated up to row H; after which the excess 100-µl mixture was discarded. The microplate was incubated at 30°C for 24 h and the bacterial growth in the wells was assessed by measuring absorbance at 600nm (OD_{600}) using Tecan Spark plate reader.

Plasmid cloning

Deletion and complementation of the *afr* locus (RPPX_14685) was performed using homologous recombination between free-ended DNA sequences that are generated by cleavage at unique I-SceI sites (23). Two homologous recombination sites were chosen downstream (TS-1) and upstream (TS-2) of the target genes. TS-1 and TS-2 fragments were obtained by performing PCR using primers listed in Table 6.6. The obtained plasmid constructs were verified by DNA sequencing. Mating was performed as described by Wynands and colleagues (25). Deletion and complementation of the *afr* locus were verified by PCR and Sanger sequencing (Macrogen B.V., Amsterdam).

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