**A *Pseudomonas putida* *cbrB* transposon insertion mutant displays a
biofilm hyperproducing phenotype that is resistant to dispersal**

Running title: Phenotypic characterization of *cbrB* mutants in *P. putida*

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SUMMARY (200 words)

The CbrAB two-component system in the Pseudomonads controls a variety of metabolic and behavioural traits required for its adaptation to changing environmental conditions, including the uptake or assimilation of certain carbon sources, and processes such as chemotaxis or stress tolerance. In this work we characterise a *miniTn5-luxAB-Km* transposon insertion mutant in *cbrB* (MPO406) in *Pseudomonas putida* leading to a biofilm overproducing phenotype that is not dispersed when nutrients are depleted. Comparison with a *cbrB* deletion mutant revealed that all phenotypes previously attributed to CbrB in *P. putida* correlated in both strains, with the exception of biofilm overproduction and absence of dispersal. We show that in the insertion mutant, the expression of the downstream regulatory RNA CrcZ is upregulated, and also show the presence of a truncated form of CbrB. Also, two additional point mutations in *lapG* and *lapD* have been detected in MPO406 by whole genome sequencing. Combination of these effects provides a robust biofilm overproducing phenotype. We present the mutant strain MPO406 as a good candidate to perform bio-production of substances of biotechnological interest or other processes such as bioremediation, which take advantage of immobilized cells on solid surfaces.

INTRODUCTION

The CbrAB two component system is exclusive of the Pseudomonads and regulates the assimilation of certain amino acids as carbon sources through the action of CbrB, a sigma N-dependent transcriptional activator similar to NtrC (Amador *et al.*, 2010, Itoh *et al.*, 2007, Li and Lu, 2007, Nishijyo *et al.*, 2001). CbrAB also controls carbon catabolite repression (CCR) and in the presence of less preferred carbon substrates, CbrB-activated transcription of the small regulatory RNAs CrcZ in *P. aeruginosa* (Sonnleitner *et al.*, 2009), and CrcZ and CrcY in *P. putida* (Garcia-Maurino *et al.*, 2013a,

Moreno *et al.*, 2012) clearly increases. This increase antagonizes the Crc/Hfq regulatory system out of its regulatory mRNA target sequences and, therefore, prevents its function (Moreno *et al.*, 2012, Moreno *et al.*, 2015). In addition, transcriptomic analysis of *P. putida* also indicated that CbrB has a regulatory role that goes beyond the use of carbon sources. This suggests that the Cbr two-component system would regulate at a high hierarchical level that may, directly or indirectly, regulate important aspects of the bacterial relationship with the environment such as chemotaxis, resistance to various toxic or even living style (Amador *et al.*, 2010).

This work presents evidence that the mutant phenotypes of a CbrB insertion mutant previously attributed to a lack of CbrB function are mostly due to lack of its activity. However, the ability to overproduce biofilm results from a side effect of the transposon inserted in *cbrB*, which results in an unusually high expression level of the downstream gene encoding the small RNA CrcZ, and in the presence of high levels of a truncated protein presumably containing the N-terminal domain of CbrB.

RESULTS AND DISCUSSION

Phenotypic characterization of *cbrB* mutant strains

The MPO406 mutant strain bears a *miniTn5-luxAB-Km* insertion that we have precisely mapped after the nucleotide 670 of *cbrB*, which corresponds to the central domain of the response regulator CbrB, where the ATPase activity resides. Using this strain we have previously reported a variety of phenotypes associated to CbrB function in *P. putida*. A very evident phenotype observed in this strain was the ability to aggregate when growing in LB liquid culture, and its ability to form very thick and persistent biofilm structures on solid surfaces (Amador *et al.*, 2010). All phenotypes previously described for the MPO406 mutant were also analysed in MPO401, an isogenic *cbrB* deletion strain previously constructed (Garcia-Maurino *et al.*, 2013b). Both mutants showed similar phenotypes

when growing in media containing different carbon sources (Table 1), they were also more resistant to copper and zinc than the wild type strain (Figure 1A), and showed reduced swimming motility (42,1 % and 48.8 % of the wild type, respectively) (Figure 1B). Also, complementation of the growth on a minimal medium with citrate as carbon source was assayed for strains MPO401 and MPO406 bearing the plasmid pMPO1250, where *cbrB* was expressed from its own promoter. In these conditions, both MPO401 and MPO406 mutant strains grew as efficiently as the wild type whereas they could not grow when bearing the empty plasmid (pBBRMCS-4) (data not shown), thus indicating that this phenotype was due to lack of CbrB.

Biofilm formation and its persistence, measured as the ability to retain crystal violet dye (O'Toole and Kolter, 1998), was also analysed for the wild type strain and MPO401 and MPO406 *cbrB* mutants in the presence or absence of CbrB supplied in *trans* (plasmid pMPO1250). In these assays, as previously described (Lopez-Sanchez *et al.*, 2013), the wild type strain initially produces biofilm (4 and 8 h) but the biofilm is dispersed after running out of nutrients (24 h) (Figure 2). When the biofilm was quantified in strain MPO406, a 3 and 3.7-fold increase of the retained biofilm was detected compared to wild type, after 4 and 8 hours post- inoculation, respectively. Additionally, unlike the wild type strain, after 24 h the biofilm in MPO406 remained intact rather than decreasing, and was 20-fold larger than that of the wild type (Figure 2). This represents a highly persistent aggregative phenotype that results in increased biofilm and pellicle formation, and flocculation. Surprisingly, the biofilm formation phenotype of the deletion strain MPO401 was the same as that of the wild type strain, although it somehow was more resilient to dispersion. The supply of *cbrB* in *trans* (plasmid pMPO1250) did not affect the ability of biofilm formation/dispersion in any of the three backgrounds (Figure 2, right), showing that CbrB does not affect biofilm formation. The resilience of the biofilm to disperse after 24

hours of incubation in the MPO406 strain is even greater than that of other biofilm-persistent mutants (i.e. *lapG*, *bifA*, *mvaB* or *dksA*), which have been described as directly involved in the process of biofilm dispersal in *P. putida* (Lopez-Sanchez *et al.*, 2013).

Reversion of the MPO406 *cbrB* insertion mutant into a *cbr*⁺ strain partially relieves the biofilm dispersion pattern

In order to certainly assign a role to the transposon insertion in MPO406 in the high biofilm production and the absence of dispersal phenotype, a reversion of the *cbrB* locus in the strain MPO406 to a wild type allele was constructed through a two-step double recombination event. For that purpose, the coding region of *cbrB* expressed under its own promoter was cloned into the suicide vector pEX18Tc, which contains the *sacB* gene conferring sucrose sensitivity. After electroporation of the resulting plasmid (pMPO1245), integration was selected by the tetracycline resistance coded by the vector. To select plasmid excision after a second recombination event, the candidates were checked for kanamycin and tetracycline sensitivity and sucrose resistance. The genomic organization of the resulting candidates was analysed by Southern Blot using probes corresponding to the *cbrB* flanking regions, and shown to have the same organization in that locus as the wild type strain (data not shown). The first important observation was that none of the revertant candidates flocculated in LB liquid culture as the MPO406 insertion strain did, thus indicating a deficiency in aggregation compared to the parental strain. The revertant strain MPO486 showing the correct genomic arrangement was assayed for biofilm formation and dispersion. The dynamics of biofilm were analysed as the alternative method to time-course measurements previously described (Lopez-Sanchez *et al.*, 2013). In this analysis, strains are serially diluted in the wells of a microtitre dish and incubated for a defined period of time prior to growth evaluation. The dilution series are shown to recapitulate the time-course of both planktonic and biofilm growth. For dilution series-

based growth curves, overnight cultures were pre-diluted in LB to an A₆₀₀ of 0.1, and then subjected to nine sixfold serial dilutions. Each of the ten diluted suspensions was used to inoculate one column of a 96-well microtitre dish. The plates were incubated for 20 to 30 h at 25°C with moderate shaking (150 r.p.m.) and processed for planktonic and biofilm growth measurement. The pattern of biofilm formation and dispersion for the KT2442, MPO401 and MPO406 after 20h of incubation before dilution (Figure 3A) recapitulated the behavior of the biofilm measured as time-course (Figure 2). The revertant MPO486 showed delayed biofilm formation and dispersion phenotype. The lag phase observed for strain MPO486 was also longer than that for the wild type and MPO406, which presumably contributed to the delayed biofilm formation and dispersion phenotype. For this reason, the dilution assay for MPO486 was performed after 30h of incubation to allow the measurement of the complete biofilm kinetics (Figure 3B). The revertant MPO486 strain was able to form a higher amount of biofilm than the wild type KT2442 strain, reaching a maximum A₆₂₀ of 1.8. This biofilm was dispersed reaching a value intermediate between WT and the insertion mutant MPO406. This result indicates that the biofilm formation/dispersion phenotype is associated to the transposon insertion. However, the revertant phenotype is not exactly as the wild type (or the CbrB deletion mutant), suggesting that there might be additional differences.

MPO406 shows high transcription of the 3' end of *cbrB* and *crcZ*

Given that the transposon insertion yielded a clear phenotype in the biofilm dynamics, the expression pattern of the adjacent genes within the same cluster was carefully analysed in order to detect other polar effects. For that purpose, quantitative analysis of *cbrB* transcription and the adjacent genes *crcZ* and *pcnB* was performed, and was compared to the wild type and strain MPO401. To validate this information, transcription within *cbrB*, at the 5' end and 3' ends of the gene (that is, upstream and downstream the transposon

1 insertion in MPO406, respectively) were also measured by RT-qPCR in the three
2 backgrounds. Although being a *cbrB*⁻ mutant, strain MPO401 bears the first 95 base pairs
3 of the coding region of the gene, thus making possible to estimate its transcription rate.
4 Quantification of the 5' end of the *cbrB* transcripts revealed that its expression was very
5 low in the wild type and strain MPO401, while the expression levels were increased 2.4
6 fold in the insertion MPO406 mutant strain (Figure 4). 5' mapping in the strains showed the
7 same transcription initiation, which was coincident to that previously shown in *P.*
8 *aeruginosa* (Nishijyo *et al.*, 2001) (data not shown), although in the mutant reached higher
9 levels of expression. This increased promoter activity was not due to the lack of CbrB
10 since the deletion mutant had the same expression levels as the wild type. However, the
11 most striking difference was detected for the expression of the 3' end of *cbrB* (downstream
12 the insertion of the transposon) in strain MPO406, which showed much higher levels (34.2
13 fold higher than in the wild type strain), suggesting spurious transcription from the
14 transposon insertion (Figure 4).

15 Regarding *crcZ*, its high transcript level in the wild type strain was reduced down to 13% in
16 the MPO401 *cbrB* deletion strain, in agreement with previous data (Garcia-Maurino *et al.*,
17 2013b) (Figure 4). Surprisingly, the *crcZ* levels in mutant MPO406 were clearly higher and
18 reached 60% of the wild type. This data suggests that there is some read-through
19 transcription from a promoter located within the transposon that yields high levels of the 3'
20 end of *cbrB* and *crcZ*. Primer extension analysis of *crcZ* in the wild type and MPO401 and
21 MPO406 genetic backgrounds confirmed that in MPO406, the 5' end of *crcZ* is not the
22 same as that of the wild type but is the result of the processing of a larger RNA species
23 due to the read-through transcription from the transposon insertion (Figure S1).

The effect of the insertion on the downstream *pcnB*, coding for a polyA polymerase, was not significant (1.1 and 1.6 fold in MPO401 and MPO406 compared to the wild type, respectively), thus suggesting that *pcnB* belongs to a different transcriptional unit.

High levels of CrcZ are not sufficient for increased biofilm formation

To test if the high levels of CrcZ could be related to the increased biofilm formation in MPO406, plasmid pMPO1249 containing *crcZ* expressed from the strong heterologous *P_{tac}* promoter was introduced in the wild type and MPO401 *cbrB* deletion backgrounds. The amount of CrcZ in strain MPO401 growing in LB increased 218-fold when extra copies of *crcZ* were delivered in *trans* [1.3 ± 0.3 arbitrary units (a.u.) with the empty vector pJB3Km1 compared to 289 ± 108 a. u. with pMPO1249]. Nevertheless, the wild type did not significantly increase any further the *crcZ* levels in the presence on the plasmid (163 ± 44 a.u. with the empty vector pJB3Km1 compared to 193 ± 40 a. u. with pMPO1249), thus suggesting the endogenous levels of the regulatory sRNA are saturating. Although a relevant increase in the amounts of CrcZ was detected, such heterologous overproduction did not cause a substantial effect on the biofilm formation either in strain MPO401 or KT2442 (data not shown), thus indicating that, in these experimental conditions, the overexpression of *crcZ* by itself was not sufficient to overproduce the biofilm or cause an effect on dispersion.

A truncated form of CbrB containing the N terminal domain, prevents biofilm dispersion in the wild type strain

To study the nature of the CbrB produced in the strain MPO406, a Western blot analysis was performed with cells growing in a minimal medium with succinate as carbon source, using polyclonal antibodies against CbrB. The wild type strain was used as a control, but CbrB (predicted molecular weight of 52.6 kDa) was not sufficiently produced to be

1 detected in this background (Figure 5). Surprisingly, a peptide with an apparent weight of
2 37 kDa was evidently detected at high level in strain MPO406. Since the transposon
3 insertion was localised at nucleotide 670 of *cbrB*, a putative N-terminal truncated CbrB
4 peptide would have a molecular weight of 29 kDa (24 kDa corresponding to CbrB plus 5
5 kDa to transposon sequence up to the next in-frame stop codon), and a putative truncated
6 peptide containing the C terminal end would be of at least 37 kDa (if there was an in-frame
7 start site within the transposon). To verify if any of the variants had an effect on biofilm
8 formation or dispersion in the wild type or revertant strain MPO496, truncated forms of
9 CbrB that would mimic the postulated CbrB fragments in MPO406 were constructed.

10 Plasmid pMPO1240 contained the 5' sequence of *cbrB* expressed from its own promoter
11 that coded for the N- terminal first 223 amino acids, which enclosed the receiver domain
12 (REC) and part of the central domain up to the transposon insertion. The DNA binding
13 domain of CbrB was missing. A sequence coding for the end of the transposon bearing its
14 putative Shine-Dalgarno sequence and the C- terminal end of *cbrB* from residue 223 was
15 cloned under a *Ptac* promoter and induced with IPTG in plasmid pMPO488. Biofilm
16 formation and dispersion was analysed as serial dilution curves for the wild type and
17 revertant MPO486 strains bearing plasmids pMPO1240 and pMPO488. Empty plasmids
18 pBBRMCS-4 and pIZ1016 were used as controls, respectively.

19 Serial dilutions curves mimicking biofilm formation and dispersion were performed for the
20 wild type and MPO486 strains in order to study the effect of over expression of a truncated
21 peptide containing the Nt (pMPO1240) or Ct (pMPO488). Over expression of the C-
22 terminal end produced no detectable effect on biofilm in either background compared to
23 the empty plasmid pIZ1016 in the presence of IPTG (data not shown). The overproduction
24 of the N-terminal truncated CbrB form did not result in a biofilm overproducing phenotype,
25 but the wild type strain containing pMPO1240 showed a clear resistance to biofilm

dispersal after 20 h of incubation, when compared to the same strain with the empty plasmid pBBRMCS-1 (Figure 6A). As a control, the wild type strain in the presence of the full length CbrB (plasmid pMPO1250) and also the empty plasmid, was also monitored, and no effect in the biofilm kinetics was detected (Figure 6B), as observed before in the time-course analysis (figure 2). Also, no appreciable effect in the biofilm dynamics was detected for strain MPO486 in the presence of the truncated N-terminal CbrB variant in plasmid pMPO1240 (data not shown). The Nt truncated version of CbrB in pMPO1240 contained the conserved aspartic residue (D52), which is subjected to phosphorylation by the histidine kinase CbrA in order to activate the response of the Cbr regulatory system, but lacked the domain for interaction with the σ^N factor of the RNA polymerase and the DNA binding domain. Therefore, production of the N-terminal regulatory domain of CbrB partially mimics the MPO406 biofilm phenotype since it is sufficient to produce a biofilm resistant to dispersal upon nutrient depletion, though not enough for the overproduction phenotype associated to the MPO406 strain. The effect of overproducing the CbrB N-terminus is probably an artifact. One possibility is that excessive phosphorylation of CbrB the N-terminus by CbrA, results in lower phosphorylation of native CbrB or other unknown regulators. However, the wild type strain overproducing it does not show the phenotypes associated to lack of Cbr function; therefore, native CbrB in this strain should be phosphorylated and active. Another possibility is that the unusually high levels of the phosphorylated CbrB N-terminus interfere with signal transduction by other two-component regulatory proteins that could control biofilm dispersal.

MPO406 strain genome sequencing reveals point mutations in biofilm-related genes

Given that the biofilm hyper-production phenotype was not fully recovered by overexpressing the truncated CbrB form, and the revertant phenotype is not exactly as the wild type one, the presence of additional chromosomal mutations in the parental strain that

was initially subjected to transposon mutagenesis to give MPO406 was assessed. For that purpose, the genomes of *P. putida* wild type strain KT2442 and MPO406 *cbrB* insertion mutant were fully sequenced. Two main differences were found in strain MPO406 compared to KT2442 that may be responsible for a biofilm phenotype. A point mutation in *lapG* (PP0164) in nucleotide 396 (C for G) leads to a substitution of a His to a Gln. Also a G to T mutation in *lapD* (PP0165) leads to a stop codon at Glu 230.

Biofilm dispersal in *P. putida* is regulated by the LapG-LapD system in response to intracellular cyclic-di-GMP (c-di-GMP) levels. LapG is a periplasmic protein that promotes biofilm dispersal through proteolysis of LapA, a high molecular weight adhesin required for initiation of biofilm formation (Gjermansen *et al.*, 2010, Gjermansen *et al.*, 2005). LapD is a transmembrane protein containing GGDEF and EAL domains that regulates LapG protease activity via direct protein-protein interactions. In the presence of high c-di-GMP levels, this effector binds the GGDEF domain of LapD to trigger a conformational change that causes the periplasmic domain of LapD to bind and inactivate LapG (Newell *et al.*, 2009), reviewed by (Boyd and O'Toole, 2012). At low c-di-GMP concentrations LapD releases LapG, thus promoting LapA proteolysis and biofilm dispersal. Accordingly, a *lapG* null mutant overproduces and fails to disperse the biofilm, due to its inability to proteolyze LapA, while a *lapD* null mutant displays constitutive LapA proteolytic activity and is therefore unable to form a biofilm (Gjermansen *et al.*, 2010, Gjermansen *et al.*, 2005). Since LapA synthesis is itself positively regulated by c-di-GMP via FleQ (Martinez-Gil *et al.*, 2014), it is feasible that biofilm growth and dispersal are ultimately controlled by the balance between c-di-GMP-induced LapA synthesis and c-di-GMP-repressed LapA proteolysis.

The presence of mutations in *lapG* and *lapD* in MPO406 (and also the revertant strain MPO486) can be reconciled with the observed biofilm growth of MPO486 in the light of the

regulatory circuit above. We propose that LapG (His132Gln) bears partial activity, while the nonsense mutation in *lapD* has a null phenotype. In this scenario, a partially active LapG protease is expected to be present at all times, regardless of the c-di-GMP levels. During biofilm growth, high c-di-GMP levels promote LapA synthesis that may titrate out the limited protease activity of LapG (His132Gln), stimulating biofilm overproduction. However, as c-di-GMP levels drop in early stationary phase, LapA synthesis is stopped, thus shifting the balance towards LapA proteolysis, hence promoting biofilm dispersal.

In this work we present a new *P. putida* derivative strain able to yield a highly persistent biofilm, which may be consequence of a double effect of point mutations on the LapD/LapG regulatory system, and the presence of a truncated form of CbrB that may interfere with some signaling pathway on the biofilm cascade. In any case, this strain supports a context that constitutes unquestionable advantages for bacterial communities such as increased stress resistance or horizontal gene transfer. In the recent years biotechnology has also exploited the properties of growth on solid media of microorganisms both in bio-production processes or decontamination or contention of pollutants (Peyton and Characklis, 1995, Singh *et al.*, 2006). Strain MPO406 constitutes a good candidate for optimization of some of these or other processes with biotechnological applications.

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TABLES

Table 1. Growth of *P. putida* KT2442 and *cbrB* mutants on different carbon sources. Growth was measured as the increase in optical density at 600 nm (ABS_{600}) in the cultures over time in *P. putida* KT2442, *P. putida* MPO406 (*cbrB*::miniTn5-*luxAB*-Km) and *P. putida* MPO401 (*cbrB*::Km) in minimal medium with ammonium as nitrogen source and Histidine, Proline, Arginine, Ornithine, Tyrosine, Glutamate, Succinate or Citrate as carbon sources; or in rich medium LB as carbon and nitrogen source. +++: $A_{600} > 0,8$ after 7 hours; ++: $A_{600} > 0,8$ after 10 hours; +: $A_{600} > 0,8$ after 15 hours; -: no growth or $A_{600} > 0,8$ after 40 hours of incubation. C: carbon, N: nitrogen.

	Source	KT2442	MPO406	MPO401
Histidine	C	+	+/-	-
Proline	C	++	+/-	+/-
Arginine	C	++	-	-
Ornithine	C	+	-	-
Tyrosine	C	+++	-	-
Glutamate	C	++	+	+
LB	C/N	+++	+++	+++
Succinate	C	+++	+++	+++
Citrate	C	+++	+/-	+/-

FIGURE LEGENDS

Figure 1. Phenotypic characterization of the strains *P. putida* KT2442, MPO406 and MPO401. (A) Resistance to heavy metals; serial dilutions (10^2 to 10^6) of each strain were plated on LB agar with no metal (left), 4 mM zinc (middle) or 5 mM copper (right) added, after incubation at 30°C for 24 hours. (B) Swimming motility assays in minimal medium semisolid agar (0.2%) containing succinate as carbon source incubated at 30°C for 24 h. A

representative picture of the motility (left) and the relative motility of the two mutant strains as compared to the wild type (right) is shown. Relative values of motility are represented normalized by those displayed by the wild-type strain, measured as the radius (in mm) of migration from the initial inoculation point. The data are mean values of 6 biological replicates, with their corresponding standard deviation. Plates were incubated at 30°C for 24 h before motility was scored.

Figure 2. Biofilm formation measured as accumulation of crystal violet dye (0.1%) and quantified at 620 nm (A_{620}) in cultures growing in LB medium in microplates after 4, 8 hours (formation) and 24 hours (dispersal). Wild type KT2442, MPO406 and MPO401 strains bearing pBBRMCS-4 empty vector (left) and pMPO1250 expressing *cbrB* (right) are shown. Bars represent the average and standard deviations of at eight technical replicas of a representative experiment

Figure 3. Representation of biofilm biomass quantification (lines) and planktonic growth (dashed lines) in dilution series-based growth curves for (A) strains KT2442 (triangles), MPO406 (squares), MPO401 (diamonds) and (B) MPO486 (circles). *Pseudomonas* overnight cultures were pre-diluted in LB to an A_{600} of 0.1, and then subjected to nine sixfold serial dilutions which was used to inoculate one column of a 96-well microtitre dish. After incubation for 20 to 30 h at 25°C with moderate shaking (150 r.p.m.), the plates are processed for planktonic (A_{600}) and biofilm growth measurement (A_{620}) for accumulation of crystal violet dye as in (Lopez-Sanchez et al., 2013). The assay was performed after 20 hours of incubation for KT2442, MPO406 and MPO401 (upper panel), and after 30 hours for MPO486 (lower panel) before dilutions. Each plot shows a representative experiment out of at least three biological replicates.

Figure 4. Expression levels of *cbrB*, *crcZ* and *pcnB* in *P. putida* KT2442, MPO406 and MPO401 strains. Quantification by RT-qPCR was performed for the 5' end of *cbrB* (black

bars; oligonucleotides *cbrB*23_1Q/ *cbrB*74_2Q), for the 3' end of *cbrB* (white bars; oligonucleotides *cbrB*_1Q/ *cbrB*_2Q), *crcZ* (grey bars; oligonucleotides *crcZ*49_1Q/ *crcZ*99_1Q) and *pcnB* (striped bars; oligonucleotides *pcnB*286_1Q/*pcnB*336_2Q), for cultures growing in a defined medium containing L-aa 0.6 mM as carbon and nitrogen sources. The values are the average of at least three independent assays. The error bars indicate the standard deviation of the means.

Figure 5. Western blotting detection of CbrB. Strains KT2442 (lane 1), MPO406 (lane 2) and MPO401 (lane 3) were grown in a minimal medium containing succinate as carbon source. As control, pure CbrB-His₆ protein (55 kDa) was used. Polyclonal antibodies against CbrB were used for detection. A CbrB peptide with low molecular weight was found in the strain MPO406, marked with an arrow.

Figure 6. Representation of biofilm biomass quantification (lines) and planktonic growth (dashed lines) in dilution series-based growth curves for (A) strain KT2442 in the presence of pMPO1240, containing the N-terminal CbrB (squares) or empty plasmid pBBRMCS4 (circles). Panel (B) shows the strain KT2442 bearing plasmid pMPO1250 with the complete *cbrB* sequence (triangles) compared to empty plasmid pBBRMCS4 (diamonds). Overnight cultures were pre-diluted in LB to an A_{600} of 0.1, and then subjected to nine sixfold serial dilutions which was used to inoculate one column of a 96-well microtitre dish. After incubation for 20 h at 25°C with moderate shaking (150 r.p.m.), the plates are processed for planktonic (A_{600}) and biofilm growth measurement (A_{620}) for accumulation of crystal violet dye as in (Lopez-Sanchez et al., 2013). Each plot shows a representative experiment out of at least three biological replicates.

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**A *Pseudomonas putida* *cbrB* transposon insertion mutant displays a
biofilm hyperproducing phenotype that is resistant to dispersal**

Running title: Phenotypic characterization of *cbrB* mutants in *P. putida*

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1 SUMMARY (200 words)

2 The CbrAB two-component system in the Pseudomonads controls a variety of
 3 metabolic and behavioural traits required for its adaptation to changing environmental
 4 conditions, including the uptake or assimilation of certain carbon sources, and processes
 5 such as chemotaxis or stress tolerance. In this work we characterise a *miniTn5-luxAB-Km*
 6 transposon insertion mutant in *cbrB* (MPO406) in *Pseudomonas putida* leading to a biofilm
 7 overproducing phenotype that is not dispersed when nutrients are depleted. Comparison
 8 with a *cbrB* deletion mutant revealed that all phenotypes previously attributed to CbrB in *P.*
 9 *putida* correlated in both strains, with the exception of biofilm overproduction and absence
 10 of dispersal. We show that in the insertion mutant, the expression of the downstream
 11 regulatory RNA CrcZ is upregulated, and also show the presence of a truncated form of
 12 CbrB. Also, two additional point mutations in *lapG* and *lapD* have been detected in
 13 MPO406 by whole genome sequencing. Combination of these effects provides a robust
 14 biofilm overproducing phenotype. We present the mutant strain MPO406 as a good
 15 candidate to perform bio-production of substances of biotechnological interest or other
 16 processes such as bioremediation, which take advantage of immobilized cells on solid
 17 surfaces.

18 INTRODUCTION

19 The CbrAB two component system is exclusive of the Pseudomonads and
 20 regulates the assimilation of certain amino acids as carbon sources through the action of
 21 of CbrB, a sigma N-dependent transcriptional activator similar to NtrC (Amador *et al.*,
 22 2010, Itoh *et al.*, 2007, Li and Lu, 2007, Nishijyo *et al.*, 2001). CbrAB also controls carbon
 23 catabolite repression (CCR) and in the presence of less preferred carbon substrates,
 24 CbrB-activated transcription of the small regulatory RNAs CrcZ in *P. aeruginosa*
 25 (Sonnleitner *et al.*, 2009), and CrcZ and CrcY in *P. putida* (Garcia-Maurino *et al.*, 2013a,

1 [Moreno et al., 2012](#)) clearly increases. This increase antagonizes the Crc/Hfq regulatory
2 system out of its regulatory mRNA target sequences and, therefore, prevents its function
3 (Moreno et al., 2012, [Moreno et al., 2015](#)). In addition, transcriptomic analysis of *P. putida*
4 also indicated that CbrB has a regulatory role that goes beyond the use of carbon sources.
5 This suggests that the Cbr two-component system would regulate at a high hierarchical
6 level that may, directly or indirectly, regulate important aspects of the bacterial relationship
7 with the environment such as chemotaxis, resistance to various toxic or even living style
8 (Amador et al., 2010).

9 This work presents evidence that the mutant phenotypes of a CbrB insertion mutant
10 previously attributed to a lack of CbrB function are mostly due to lack of its activity.
11 However, the ability to overproduce biofilm results from a side effect of the transposon
12 inserted in *cbrB*, which results in an unusually high expression level of the downstream
13 gene encoding the small RNA CrcZ, and in the presence of high levels of a truncated
14 protein presumably containing the N-terminal domain of CbrB.

15 RESULTS AND DISCUSSION

16 Phenotypic characterization of *cbrB* mutant strains

17 The MPO406 mutant strain bears a *miniTn5-luxAB-Km* insertion that we have precisely
18 mapped after the nucleotide 670 of *cbrB*, which corresponds to the central domain of the
19 response regulator CbrB, where the ATPase activity resides. Using this strain we have
20 previously reported a variety of phenotypes associated to CbrB function in *P. putida*. A
21 very evident phenotype observed in this strain was the ability to aggregate when growing
22 in LB liquid culture, and its ability to form very thick and persistent biofilm structures on
23 solid surfaces (Amador et al., 2010). All phenotypes previously described for the MPO406
24 mutant were also analysed in MPO401, an isogenic *cbrB* deletion strain previously
25 constructed (Garcia-Maurino et al., 2013b). Both mutants showed similar phenotypes

1 when growing in media containing different carbon sources (Table 1), they were also more
2 resistant to copper and zinc than the wild type strain (Figure 1A), and showed reduced
3 swimming motility (42,1 % and 48.8 % of the wild type, respectively) (Figure 1B). Also,
4 complementation of the growth on a minimal medium with citrate as carbon source was
5 assayed for strains MPO401 and MPO406 bearing the plasmid pMPO1250, where *cbrB*
6 was expressed from its own promoter. In these conditions, both MPO401 and MPO406
7 mutant strains grew as efficiently as the wild type whereas they could not grow when
8 bearing the empty plasmid (pBBRMCS-4) (data not shown), thus indicating that this
9 phenotype was due to lack of CbrB.

10 Biofilm formation and its persistence, measured as the ability to retain crystal violet dye
11 (O'Toole and Kolter, 1998), was also analysed for the wild type strain and MPO401 and
12 MPO406 *cbrB* mutants in the presence or absence of CbrB supplied in *trans* (plasmid
13 pMPO1250). In these assays, as previously described (Lopez-Sanchez *et al.*, 2013), the
14 wild type strain initially produces biofilm (4 and 8 h) but the biofilm is dispersed after
15 running out of nutrients (24 h) (Figure 2). When the biofilm was quantified in strain
16 MPO406, a 3 and 3.7-fold increase of the retained biofilm was detected compared to wild
17 type, after 4 and 8 hours post- inoculation, respectively. Additionally, unlike the wild type
18 strain, after 24 h the biofilm in MPO406 remained intact rather than decreasing, and was
19 20-fold larger than that of the wild type (Figure 2). This represents a highly persistent
20 aggregative phenotype that results in increased biofilm and pellicle formation, and
21 flocculation. Surprisingly, the biofilm formation phenotype of the deletion strain MPO401
22 was the same as that of the wild type strain, although it somehow was more resilient to
23 dispersion. The supply of *cbrB* in *trans* (plasmid pMPO1250) did not affect the ability of
24 biofilm formation/dispersion in any of the three backgrounds (Figure 2, right), showing that
25 CbrB does not affect biofilm formation. The resilience of the biofilm to disperse after 24

1 hours of incubation in the MPO406 strain is even greater than that of other biofilm-
2 persistent mutants (i.e. *lapG*, *bifA*, *mvaB* or *dksA*), which have been described as directly
3 involved in the process of biofilm dispersal in *P. putida* (Lopez-Sanchez *et al.*, 2013).

4 **Reversion of the MPO406 *cbrB* insertion mutant into a *cbr*⁺ strain partially relieves** 5 **the biofilm dispersion pattern**

6 In order to certainly assign a role to the transposon insertion in MPO406 in the high biofilm
7 production and the absence of dispersal phenotype, a reversion of the *cbrB* locus in the
8 strain MPO406 to a wild type allele was constructed through a two-step double
9 recombination event. For that purpose, the coding region of *cbrB* expressed under its own
10 promoter was cloned into the suicide vector pEX18Tc, which contains the *sacB* gene
11 conferring sucrose sensitivity. After electroporation of the resulting plasmid (pMPO1245),
12 integration was selected by the tetracycline resistance coded by the vector. To select
13 plasmid excision after a second recombination event, the candidates were checked for
14 kanamycin and tetracycline sensitivity and sucrose resistance. The genomic organization
15 of the resulting candidates was analysed by Southern Blot using probes corresponding to
16 the *cbrB* flanking regions, and shown to have the same organization in that locus as the
17 wild type strain (data not shown). The first important observation was that none of the
18 revertant candidates flocculated in LB liquid culture as the MPO406 insertion strain did,
19 thus indicating a deficiency in aggregation compared to the parental strain. The revertant
20 strain MPO486 showing the correct genomic arrangement was assayed for biofilm
21 formation and dispersion. The dynamics of biofilm were analysed as the alternative
22 method to time-course measurements previously described (Lopez-Sanchez *et al.*, 2013).
23 In this analysis, strains are serially diluted in the wells of a microtitre dish and incubated for
24 a defined period of time prior to growth evaluation. The dilution series are shown to
25 recapitulate the time-course of both planktonic and biofilm growth. For dilution series-

1 based growth curves, overnight cultures were pre-diluted in LB to an A₆₀₀ of 0.1, and then
 2 subjected to nine sixfold serial dilutions. Each of the ten diluted suspensions was used to
 3 inoculate one column of a 96-well microtitre dish. The plates were incubated for 20 to 30 h
 4 at 25°C with moderate shaking (150 r.p.m.) and processed for planktonic and biofilm
 5 growth measurement. The pattern of biofilm formation and dispersion for the KT2442,
 6 MPO401 and MPO406 after 20h of incubation before dilution (Figure 3A) recapitulated the
 7 behavior of the biofilm measured as time-course (Figure 2). The revertant MPO486
 8 showed delayed biofilm formation and dispersion phenotype. The lag phase observed for
 9 strain MPO486 was also longer than that for the wild type and MPO406, which presumably
 10 contributed to the delayed biofilm formation and dispersion phenotype. For this reason, the
 11 dilution assay for MPO486 was performed after 30h of incubation to allow the
 12 measurement of the complete biofilm kinetics (Figure 3B). The revertant MPO486 strain
 13 was able to form a higher amount of biofilm than the wild type KT2442 strain, reaching a
 14 maximum A₆₂₀ of 1.8. This biofilm was dispersed reaching a value intermediate between
 15 WT and the insertion mutant MPO406. This result indicates that the biofilm
 16 formation/dispersion phenotype is associated to the transposon insertion. However, the
 17 revertant phenotype is not exactly as the wild type (or the *CbrB* deletion mutant),
 18 suggesting that there might be additional differences.

19 **MPO406 shows high transcription of the 3' end of *cbrB* and *crcZ***

20 Given that the transposon insertion yielded a clear phenotype in the biofilm dynamics, the
 21 expression pattern of the adjacent genes within the same cluster was carefully analysed in
 22 order to detect other polar effects. For that purpose, quantitative analysis of *cbrB*
 23 transcription and the adjacent genes *crcZ* and *pcnB* was performed, and was compared to
 24 the wild type and strain MPO401. To validate this information, transcription within *cbrB*, at
 25 the 5' end and 3' ends of the gene (that is, upstream and downstream the transposon

1 insertion in MPO406, respectively) were also measured by RT-qPCR in the three
2 backgrounds. Although being a *cbrB*⁻ mutant, strain MPO401 bears the first 95 base pairs
3 of the coding region of the gene, thus making possible to estimate its transcription rate.
4 Quantification of the 5' end of the *cbrB* transcripts revealed that its expression was very
5 low in the wild type and strain MPO401, while the expression levels were increased 2.4
6 fold in the insertion MPO406 mutant strain (Figure 4). 5' mapping in the strains showed the
7 same transcription initiation, which was coincident to that previously shown in *P.*
8 *aeruginosa* (Nishijyo *et al.*, 2001) (data not shown), although in the mutant reached higher
9 levels of expression. This increased promoter activity was not due to the lack of CbrB
10 since the deletion mutant had the same expression levels as the wild type. However, the
11 most striking difference was detected for the expression of the 3' end of *cbrB* (downstream
12 the insertion of the transposon) in strain MPO406, which showed much higher levels (34.2
13 fold higher than in the wild type strain), suggesting spurious transcription from the
14 transposon insertion (Figure 4).

15 Regarding *crcZ*, its high transcript level in the wild type strain was reduced down to 13% in
16 the MPO401 *cbrB* deletion strain, in agreement with previous data (Garcia-Maurino *et al.*,
17 2013b) (Figure 4). Surprisingly, the *crcZ* levels in mutant MPO406 were clearly higher and
18 reached 60% of the wild type. This data suggests that there is some read-through
19 transcription from a promoter located within the transposon that yields high levels of the 3'
20 end of *cbrB* and *crcZ*. Primer extension analysis of *crcZ* in the wild type and MPO401 and
21 MPO406 genetic backgrounds confirmed that in MPO406, the 5' end of *crcZ* is not the
22 same as that of the wild type but is the result of the processing of a larger RNA species
23 due to the read-through transcription from the transposon insertion (Figure S1).

1 The effect of the insertion on the downstream *pcnB*, coding for a polyA polymerase, was
2 not significant (1.1 and 1.6 fold in MPO401 and MPO406 compared to the wild type,
3 respectively), thus suggesting that *pcnB* belongs to a different transcriptional unit.

4 **High levels of CrcZ are not sufficient for increased biofilm formation**

5 To test if the high levels of CrcZ could be related to the increased biofilm formation in
6 MPO406, plasmid pMPO1249 containing *crcZ* expressed from the strong heterologous
7 *Ptac* promoter was introduced in the wild type and MPO401 *cbrB* deletion backgrounds.
8 The amount of CrcZ in strain MPO401 growing in LB increased 218-fold when extra copies
9 of *crcZ* were delivered in *trans* [1.3 ± 0.3 arbitrary units (a.u.) with the empty vector
10 pJB3Km1 compared to 289 ± 108 a. u. with pMPO1249]. Nevertheless, the wild type did
11 not significantly increase any further the *crcZ* levels in the presence on the plasmid ($163 \pm$
12 44 a.u. with the empty vector pJB3Km1 compared to 193 ± 40 a. u. with pMPO1249), thus
13 suggesting the endogenous levels of the regulatory sRNA are saturating. Although a
14 relevant increase in the amounts of CrcZ was detected, such heterologous overproduction
15 did not cause a substantial effect on the biofilm formation either in strain MPO401 or
16 KT2442 (data not shown), thus indicating that, in these experimental conditions, the
17 overexpression of *crcZ* by itself was not sufficient to overproduce the biofilm or cause an
18 effect on dispersion.

19 **A truncated form of CbrB containing the N terminal domain, prevents biofilm** 20 **dispersion in the wild type strain**

21 To study the nature of the CbrB produced in the strain MPO406, a Western blot analysis
22 was performed with cells growing in a minimal medium with succinate as carbon source,
23 using polyclonal antibodies against CbrB. The wild type strain was used as a control, but
24 CbrB (predicted molecular weight of 52.6 kDa) was not sufficiently produced to be

1 detected in this background (Figure 5). Surprisingly, a peptide with an apparent weight of
2 37 kDa was evidently detected at high level in strain MPO406. Since the transposon
3 insertion was localised at nucleotide 670 of *cbrB*, a putative N-terminal truncated CbrB
4 peptide would have a molecular weight of 29 kDa (24 kDa corresponding to CbrB plus 5
5 kDa to transposon sequence up to the next in-frame stop codon), and a putative truncated
6 peptide containing the C terminal end would be of at least 37 kDa (if there was an in-frame
7 start site within the transposon). To verify if any of the variants had an effect on biofilm
8 formation or dispersion in the wild type or revertant strain MPO496, truncated forms of
9 CbrB that would mimic the postulated CbrB fragments in MPO406 were constructed.

10 Plasmid pMPO1240 contained the 5' sequence of *cbrB* expressed from its own promoter
11 that coded for the N- terminal first 223 amino acids, which enclosed the receiver domain
12 (REC) and part of the central domain up to the transposon insertion. The DNA binding
13 domain of CbrB was missing. A sequence coding for the end of the transposon bearing its
14 putative Shine-Dalgarno sequence and the C- terminal end of *cbrB* from residue 223 was
15 cloned under a *Ptac* promoter and induced with IPTG in plasmid pMPO488. Biofilm
16 formation and dispersion was analysed as serial dilution curves for the wild type and
17 revertant MPO486 strains bearing plasmids pMPO1240 and pMPO488. Empty plasmids
18 pBBRMCS-4 and pIZ1016 were used as controls, respectively.

19 Serial dilutions curves mimicking biofilm formation and dispersion were performed for the
20 wild type and MPO486 strains in order to study the effect of over expression of a truncated
21 peptide containing the Nt (pMPO1240) or Ct (pMPO488). Over expression of the C-
22 terminal end produced no detectable effect on biofilm in either background compared to
23 the empty plasmid pIZ1016 in the presence of IPTG (data not shown). The overproduction
24 of the N-terminal truncated CbrB form did not result in a biofilm overproducing phenotype,
25 but the wild type strain containing pMPO1240 showed a clear resistance to biofilm

1 dispersal after 20 h of incubation, when compared to the same strain with the empty
 2 plasmid pBBRMCS-1 (Figure 6A). As a control, the wild type strain in the presence of the
 3 full length CbrB (plasmid pMPO1250) and also the empty plasmid, was also monitored,
 4 and no effect in the biofilm kinetics was detected (Figure 6B), as observed before in the
 5 time-course analysis (figure 2). Also, no appreciable effect in the biofilm dynamics was
 6 detected for strain MPO486 in the presence of the truncated N-terminal CbrB variant in
 7 plasmid pMPO1240 (data not shown). The Nt truncated version of CbrB in pMPO1240
 8 contained the conserved aspartic residue (D52), which is subjected to phosphorylation by
 9 the histidine kinase CbrA in order to activate the response of the Cbr regulatory system,
 10 but lacked the domain for interaction with the σ^N factor of the RNA polymerase and the
 11 DNA binding domain. Therefore, production of the N-terminal regulatory domain of CbrB
 12 partially mimics the MPO406 biofilm phenotype since it is sufficient to produce a biofilm
 13 resistant to dispersal upon nutrient depletion, though not enough for the overproduction
 14 phenotype associated to the MPO406 strain. The effect of overproducing the CbrB N-
 15 terminus is probably an artifact. One possibility is that excessive phosphorylation of CbrB
 16 the N-terminus by CbrA, results in lower phosphorylation of native CbrB or other unknown
 17 regulators. However, the wild type strain overproducing it does not show the phenotypes
 18 associated to lack of Cbr function; therefore, native CbrB in this strain should be
 19 phosphorylated and active. Another possibility is that the unusually high levels of the
 20 phosphorylated CbrB N-terminus interfere with signal transduction by other two-
 21 component regulatory proteins that could control biofilm dispersal.

22 **MPO406 strain genome sequencing reveals point mutations in biofilm-related genes**

23 Given that the biofilm hyper-production phenotype was not fully recovered by
 24 overexpressing the truncated CbrB form, and the revertant phenotype is not exactly as the
 25 wild type one, the presence of additional chromosomal mutations in the parental strain that

1 was initially subjected to transposon mutagenesis to give MPO406 was assessed. For that
2 purpose, the genomes of *P. putida* wild type strain KT2442 and MPO406 *cbrB* insertion
3 mutant were fully sequenced. Two main differences were found in strain MPO406
4 compared to KT2442 that may be responsible for a biofilm phenotype. A point mutation in
5 *lapG* (PP0164) in nucleotide 396 (C for G) leads to a substitution of a His to a Gln. Also a
6 G to T mutation in *lapD* (PP0165) leads to a stop codon at Glu 230.

7 Biofilm dispersal in *P. putida* is regulated by the LapG-LapD system in response to
8 intracellular cyclic-di-GMP (c-di-GMP) levels. LapG is a periplasmic protein that promotes
9 biofilm dispersal through proteolysis of LapA, a high molecular weight adhesin required for
10 initiation of biofilm formation (Gjermansen *et al.*, 2010, Gjermansen *et al.*, 2005). LapD is a
11 transmembrane protein containing GGDEF and EAL domains that regulates LapG
12 protease activity via direct protein-protein interactions. In the presence of high c-di-GMP
13 levels, this effector binds the GGDEF domain of LapD to trigger a conformational change
14 that causes the periplasmic domain of LapD to bind and inactivate LapG (Newell *et al.*,
15 2009), reviewed by (Boyd and O'Toole, 2012). At low c-di-GMP concentrations LapD
16 releases LapG, thus promoting LapA proteolysis and biofilm dispersal. Accordingly, a *lapG*
17 null mutant overproduces and fails to disperse the biofilm, due to its inability to proteolyze
18 LapA, while a *lapD* null mutant displays constitutive LapA proteolytic activity and is
19 therefore unable to form a biofilm (Gjermansen *et al.*, 2010, Gjermansen *et al.*, 2005).
20 Since LapA synthesis is itself positively regulated by c-di-GMP via FleQ (Martinez-Gil *et*
21 *al.*, 2014), it is feasible that biofilm growth and dispersal are ultimately controlled by the
22 balance between c-di-GMP-induced LapA synthesis and c-di-GMP-repressed LapA
23 proteolysis.

24 The presence of mutations in *lapG* and *lapD* in MPO406 (and also the revertant strain
25 MPO486) can be reconciled with the observed biofilm growth of MPO486 in the light of the

1 regulatory circuit above. We propose that LapG (His132Gln) bears partial activity, while
 2 the nonsense mutation in *lapD* has a null phenotype. In this scenario, a partially active
 3 LapG protease is expected to be present at all times, regardless of the c-di-GMP levels.
 4 During biofilm growth, high c-di-GMP levels promote LapA synthesis that may titrate out the
 5 limited protease activity of LapG (His132Gln), stimulating biofilm overproduction. However,
 6 as c-di-GMP levels drop in early stationary phase, LapA synthesis is stopped, thus shifting
 7 the balance towards LapA proteolysis, hence promoting biofilm dispersal.

8 In this work we present a new *P. putida* derivative strain able to yield a highly persistent
 9 biofilm, which may be consequence of a double effect ~~on~~-of point mutations on the
 10 LapD/LapG regulatory system, and the presence of a truncated form of CbrB that may
 11 interfere with some signaling pathway on the biofilm cascade. In any case, this strain
 12 supports a context that constitutes unquestionable advantages for bacterial communities
 13 such as increased stress resistance or horizontal gene transfer. In the recent years
 14 biotechnology has also exploited the properties of growth on solid media of
 15 microorganisms both in bio-production processes or decontamination or contention of
 16 pollutants (Peyton and Characklis, 1995, Singh *et al.*, 2006). Strain MPO406 constitutes a
 17 good candidate for optimization of some of these or other processes with biotechnological
 18 applications.

19

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12

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TABLES

Table 1. Growth of *P. putida* KT2442 and *cbrB* mutants on different carbon sources. Growth was measured as the increase in optical density at 600 nm (ABS_{600}) in the cultures over time in *P. putida* KT2442, *P. putida* MPO406 (*cbrB*::miniTn5-*luxAB*-Km) and *P. putida* MPO401 (*cbrB*::Km) in minimal medium with ammonium as nitrogen source and Histidine, Proline, Arginine, Ornithine, Tyrosine, Glutamate, Succinate or Citrate as carbon sources; or in rich medium LB as carbon and nitrogen source. +++: $A_{600} > 0,8$ after 7 hours; ++: $A_{600} > 0,8$ after 10 hours; +: $A_{600} > 0,8$ after 15 hours; -: no growth or $A_{600} > 0,8$ after 40 hours of incubation. C: carbon, N: nitrogen.

	Source	KT2442	MPO406	MPO401
Histidine	C	+	+/-	-
Proline	C	++	+/-	+/-
Arginine	C	++	-	-
Ornithine	C	+	-	-
Tyrosine	C	+++	-	-
Glutamate	C	++	+	+
LB	C/N	+++	+++	+++
Succinate	C	+++	+++	+++
Citrate	C	+++	+/-	+/-

FIGURE LEGENDS

Figure 1. Phenotypic characterization of the strains *P. putida* KT2442, MPO406 and MPO401. (A) Resistance to heavy metals; serial dilutions (10^2 to 10^6) of each strain were plated on LB agar with no metal (left), 4 mM zinc (middle) or 5 mM copper (right) added, after incubation at 30°C for 24 hours. (B) Swimming motility assays in minimal medium semisolid agar (0.2%) containing succinate as carbon source incubated at 30°C for 24 h. A

representative picture of the motility (left) and the relative motility of the two mutant strains as compared to the wild type (right) is shown. Relative values of motility are represented normalized by those displayed by the wild-type strain, measured as the radius (in mm) of migration from the initial inoculation point. The data are mean values of 6 biological replicates, with their corresponding standard deviation. Plates were incubated at 30°C for 24 h before motility was scored.

Figure 2. Biofilm formation measured as accumulation of crystal violet dye (0.1%) and quantified at 620 nm (A_{620}) in cultures growing in LB medium in microplates after 4, 8 hours (formation) and 24 hours (dispersal). Wild type KT2442, MPO406 and MPO401 strains bearing pBBRMCS-4 empty vector (left) and pMPO1250 expressing *cbrB* (right) are shown. Bars represent the average and standard deviations of at eight technical replicas of a representative experiment

Figure 3. Representation of biofilm biomass quantification (lines) and planktonic growth (dashed lines) in dilution series-based growth curves for (A) strains KT2442 (triangles), MPO406 (squares), MPO401 (diamonds) and (B) MPO486 (circles). *Pseudomonas* overnight cultures were pre-diluted in LB to an A_{600} of 0.1, and then subjected to nine sixfold serial dilutions which was used to inoculate one column of a 96-well microtitre dish. After incubation for 20 to 30 h at 25°C with moderate shaking (150 r.p.m.), the plates are processed for planktonic (A_{600}) and biofilm growth measurement (A_{620}) for accumulation of crystal violet dye as in (Lopez-Sanchez et al., 2013). The assay was performed after 20 hours of incubation for KT2442, MPO406 and MPO401 (upper panel), and after 30 hours for MPO486 (lower panel) before dilutions. Each plot shows a representative experiment out of at least three biological replicates.

Figure 4. Expression levels of *cbrB*, *crcZ* and *pcnB* in *P. putida* KT2442, MPO406 and MPO401 strains. Quantification by RT-qPCR was performed for the 5' end of *cbrB* (black

bars; oligonucleotides *cbrB*23_1Q/ *cbrB*74_2Q), for the 3' end of *cbrB* (white bars; oligonucleotides *cbrB*_1Q/ *cbrB*_2Q), *crcZ* (grey bars; oligonucleotides *crcZ*49_1Q/ *crcZ*99_1Q) and *pcnB* (striped bars; oligonucleotides *pcnB*286_1Q/*pcnB*336_2Q), for cultures growing in a defined medium containing L-aa 0.6 mM as carbon and nitrogen sources. The values are the average of at least three independent assays. The error bars indicate the standard deviation of the means.

Figure 5. Western blotting detection of CbrB. Strains KT2442 (lane 1), MPO406 (lane 2) and MPO401 (lane 3) were grown in a minimal medium containing succinate as carbon source. As control, pure CbrB-His₆ protein (55 kDa) was used. Polyclonal antibodies against CbrB were used for detection. A CbrB peptide with low molecular weight was found in the strain MPO406, marked with an arrow.

Figure 6. Representation of biofilm biomass quantification (lines) and planktonic growth (dashed lines) in dilution series-based growth curves for (A) strain KT2442 in the presence of pMPO1240, containing the N-terminal CbrB (squares) or empty plasmid pBBRMCS4 (circles). Panel (B) shows the strain KT2442 bearing plasmid pMPO1250 with the complete *cbrB* sequence (triangles) compared to empty plasmid pBBRMCS4 (diamonds). Overnight cultures were pre-diluted in LB to an A_{600} of 0.1, and then subjected to nine sixfold serial dilutions which was used to inoculate one column of a 96-well microtitre dish. After incubation for 20 h at 25°C with moderate shaking (150 r.p.m.), the plates are processed for planktonic (A_{600}) and biofilm growth measurement (A_{620}) for accumulation of crystal violet dye as in (Lopez-Sanchez et al., 2013). Each plot shows a representative experiment out of at least three biological replicates.

Fig1
67x57mm (300 x 300 DPI)

Fig2
48x28mm (300 x 300 DPI)

Fig3
107x144mm (300 x 300 DPI)

Fig4
60x45mm (300 x 300 DPI)

Fig5
38x24mm (300 x 300 DPI)

Fig6
128x206mm (300 x 300 DPI)