Novel Targets of the CbrAB/Crc Carbon Catabolite Control System Revealed by Transcript Abundance in 
*Pseudomonas aeruginosa*

Elisabeth Sonnleitner1*, Martina Valentini2, Nicolas Wenner2, Feth el Zahar Haichar2*, Dieter Haas2, Karine Lapouge2*

1 Max F. Perutz Laboratories, Department of Microbiology, Immunobiology and Genetics, University of Vienna, Dr. Bohrgasse 9, Vienna, Austria, 2Department of Fundamental Microbiology, University of Lausanne, Lausanne, Switzerland

### Abstract

The opportunistic human pathogen *Pseudomonas aeruginosa* is able to utilize a wide range of carbon and nitrogen compounds, allowing it to grow in vastly different environments. The uptake and catabolism of growth substrates are organized hierarchically by a mechanism termed catabolite repression control (Crc) whereby the Crc protein establishes translational repression of target mRNAs at CA (catabolite activity) motifs present in target mRNAs near ribosome binding sites. Poor carbon sources lead to activation of the CbrAB two-component system, which induces transcription of the small RNA (sRNA) CrcZ. This sRNA relieves Crc-mediated repression of target mRNAs. In this study, we have identified novel targets of the CbrAB/Crc system in *P. aeruginosa* using transcriptome analysis in combination with a search for CA motifs. We characterized four target genes involved in the uptake and utilization of less preferred carbon sources: estA (secreted esterase), acsA (acetyl-CoA synthetase), bdkR (regulator of branched-chain amino acid catabolism) and aroP2 (aromatic amino acid uptake protein). Evidence for regulation by CbrAB/CrcZ and Crc was obtained in vivo using appropriate reporter fusions, in which mutation of the CA motif resulted in loss of catabolite repression. CbrB and CrcZ were important for growth of *P. aeruginosa* in cystic fibrosis (CF) sputum medium, suggesting that the CbrB/Crc system may act as an important regulator during chronic infection of the CF lung.

### Introduction

*Pseudomonas aeruginosa* is a metabolically versatile organism, which is able to grow in a variety of different niches. Its natural habitats can be nutrient-poor (e.g., aqueous environments) or nutrient-rich (e.g., the rhizosphere or infected animal tissues). The CbrAB/Crc carbon catabolite control system helps to ensure a metabolic optimization process by allowing preferential utilization of good energy sources and establishing a healthy carbon (C) to nitrogen (N) balance [1]. The CbrAB/Crc system involves a regulatory protein (Crc), which is required to establish translational repression of target mRNAs at CA (catabolite activity) motifs, usually located in the vicinity of ribosome binding sites [1–4]. The core sequence of the CA motif consists of eight nucleotides, AAnAAnAA, where n stands for any nucleotide. However, inspection of RNAs that are under Crc control suggests that n = C or U may be preferred over n = A or G in CA motifs [1,2]. A small RNA (sRNA) termed CrcZ, which possesses several conserved CA motifs and appears to bind to Crc, antagonizes translational repression mediated by the Crc protein [1]. CrcZ levels are low in the presence of a good carbon source (e.g., succinate), elevated in the presence of an intermediate carbon source (e.g., glucose) and high when a less preferred carbon compound is used as the sole energy source (e.g., mannitol). This regulation is established by the activity of the two-component system CbrAB consisting of the membrane-bound sensor CbrA and the response regulator CbrB, which are required for the expression of *acrZ*, together with the alternative sigma factor RpoN and IHF protein [1,5]. CbrB binds to an upstream activating sequence (UAS) in the *acrZ* promoter region [5]. Mutants affected in *cbrB* or *acrZ* have similar, but not identical phenotypes, indicating that CrcZ mediates most, but not all, output of the CbrAB system [1,5]. The CbrAB/Crc system is involved in the regulation of swarming, biofilm formation, cytotoxicity and antibiotic resistance in *P. aeruginosa* [6,7]. However, in these interactions the regulatory mechanisms have not been investigated in molecular detail.

While the effects of the CbrB/Crc system occur essentially at a post-transcriptional level, we asked whether transcript abundance could be used to reveal targets of this system. Previous experience with the mechanistically similar GacSA/
RsmA pathway in *P. aeruginosa* [8–10] and *P. fluorescens* Pf-5 [11] suggests that during translational repression transcript abundance tends to be low, whereas transcript levels may be elevated during derepression, presumably reflecting effects of translational control on mRNA stability. Based on these observations, we decided to compare the transcriptome of the *P. aeruginosa* wild type PAO1 with that of its *crc*, *crcZ* and *cbrB* mutants. To find new targets of the CbrAB/Crc system, we focused on several mRNAs having appropriately located CA motifs. The targets chosen are all involved in the utilization of relatively poor C sources. Furthermore, we found that growth of *cbrB* and *crcZ* mutants was severely impaired in an artificial cystic fibrosis (CF) sputum medium, an effect that is most likely due to repression of amino acid uptake and catabolism by the Crc protein. Thus, the CbrAB/Crc system may be an important regulatory system enabling *P. aeruginosa* to thrive in the CF lung.

**Results**

**Transcriptome analysis of genes regulated by Crc** – To assess the impact of the CbrAB/Crc pathway on global gene expression of *P. aeruginosa*, we performed a transcriptome analysis of *P. aeruginosa* PAO1 wild type and its *crc*, *crcZ* and *cbrB* deletion mutants using commercially available genome-wide DNA microarrays (Affymetrix). First, we grew cells to late exponential phase in Luria Broth (LB) as a nutrient rich medium, which mainly contains amino acids, peptides and small amounts of sugars and nucleotides [12] and which creates conditions of moderate carbon catabolite repression [3]. Second, we used a defined basal salts medium (BSM) amended with 40 mM succinate, which establishes strong catabolite repression in strain PAO1 [1]. Under both conditions, Crc mediates catabolite repression of *amiE*, which encodes short-chain aliphatic amidase and has previously served as a reporter gene ([1]; Figure S1). We expected that genes whose expression is translationally repressed by the CbrAB/Crc system might show elevated transcript levels in a crc mutant, whereas down-regulated transcript levels might be seen in both cbrB and *crcZ* mutants, due arrest of translation.

In LB-grown cells, we found 57 transcripts to be differentially (≥2.0-fold) expressed in the *crc* mutant, by comparison with the wild type PAO1; 19 transcripts were up-regulated and 38 were down-regulated at least two-fold (Table S1). After growth in BSM containing succinate, 95 transcripts showed altered expression; 53 transcripts were up-regulated and 42 were down-regulated (Table S1). The overlap of genes that were differentially affected under both conditions was small (Figure 1A). The probable reason is that transcriptional expression of catabolic genes varies greatly between the two media, making it difficult to detect the same effects of Crc on transcript abundance. Nevertheless, the combined data from both media enabled us to see a considerable number of transcripts influenced by Crc. As a control, we checked *amiE* transcript levels. They were elevated in the Δcrc mutant cultivated in LB (Table 1 and Table S1), which is consistent with increased *amiE* translation (Figure S1) and, presumably, with concomitantly enhanced *amiE* mRNA stability in the absence of Crc-mediated catabolite repression. By contrast, the Δcrc mutation had no effect on *amiE* transcript abundance in cells grown in BSM, which lacks inducing amides and hence does not permit transcriptional expression of the *amiE* operon [1,13]. These results suggest that transcriptome analysis may be used to identify targets of the CbrAB/Crc system provided that sufficient transcriptional expression of the target genes takes place.

Transcriptome analysis may reveal both and indirect effects of the CbrAB/Crc system. Direct effects are assumed to involve at least one CA motif (AAnAAnAA) in the region of −50 to +50 nucleotides relative to the translational start site in target mRNAs. We found 29 Crc-regulated transcripts having an appropriately located putative CA motif (Table S1). With the exception of four transcripts (three of them in LB and one in BSM), all mRNAs harbouring a CA motif were up-regulated in the crc mutant.

**Transcriptome analysis of crcZ and cbrB mutants** – As mentioned above, the Crc protein and the CrcZ sRNA, which requires CbrB to be transcribed, have opposite regulatory effects; thus one might expect that transcripts that are up-regulated in a *crc* mutant would be down-regulated in *crcZ* and *cbrB* mutants and vice versa. To investigate this assumption, we performed transcriptome analyses with a *crcZ* and a *cbrB* mutant under the same conditions as above. In LB-grown cells, 145 transcripts were differentially expressed by a factor of ≥2.0 in PAO1Δ*crcZ*, whereas 166 transcripts were differentially expressed in PAO1Δ*cbrB*, by comparison with the wild type PAO1 (Table S2). In BSM-grown cells, 157 transcripts showed differential regulation in the *crcZ* mutant and 60 transcripts in the *cbrB* mutant (Table S2). Again, the number of genes showing a similar expression pattern in both media was low (Figure 1B and 1C). However, the *crcZ* and *cbrB* mutations had largely parallel effects; both mutations affected 101 out of 210 transcripts and 59 out of 158 transcripts in LB (Figure 1D) and in BSM (Figure 1E), respectively. Among the transcripts that were affected by both mutations, a majority was down-regulated (Table S2). Some transcripts seemed to be only regulated by CbrB and not by CrcZ. However, preliminary attempts to identify CbrB binding sites upstream of the corresponding ORFs have not been successful. Apart from the *crcZ* promoter, known direct CbrB targets include the *lipA* and *hutU* promoters [5,14]. However, these genes did not appear in our transcriptome analysis, presumably because of lack of transcriptional inducers in the growth media. Some transcripts whose abundance appeared to be only regulated by CrcZ, but not by CbrB, were also seen, but were considered as questionable since *crcZ* expression strictly depends on CrcB [1]. Taken together, our transcriptome analysis is consistent with the current model stating that the sRNA CrcZ mediates most effects of the CbrAB two-component system.

Somewhat unexpectedly, the overlap between transcripts whose abundance was affected by Crc as well as by CrcZ and/or CbrB in an opposite manner was low in BSM-grown cells (17 out of 47 genes; Figures 1D and 1E, Table 1) and absent in LB-grown cells. Among the genes that did show the anticipated transcript pattern were *estA* (encoding a secreted esterase), the *dctP* promoter (required for *Ccr*-dicarboxylate transport at low concentrations), *braC* and the *braDEFG* operon (which are important for transport of branched chain amino acids) (Table 1). Moreover, *estA*, *dctP* and *braC* possess CA motifs indicating that they may be targets of the CbrAB/Crc two-component system.

Transcripts that were strongly up-regulated in LB in the absence of Crc, but not down-regulated in the absence of CrcZ or CbrB, included *opdH* (encoding a c-acetoin porin) and *aroP2* (encoding an aromatic amino acid transporter) (Table 1). For *opdH* and *aroP2* candidate CA motifs were found. Transcripts that were strongly up-regulated in BSM in the absence of Crc included *acaA* (encoding acetyl-CoA synthetase required for acetate catabolism), *opdH*, *braC*, *braDEFG*, *bkdA1* (first gene of an operon in branched-chain amino acid assimilation), *aocR* and *acaAB* (for acetoin catabolism). CA motifs were found in all of these transcripts except for *braDEFG* and *bkdA1* (Table 1), which provides evidence that these genes may be targets of the CbrAB/Crc system.

Some striking effects of the CbrAB/Crc system on the transcriptome were not associated with CA motifs. For example,
the pqqABCDE and phnAB operons were strongly down-regulated in the \( \text{crc} \) \( \text{Z} \) and \( \text{cbrB} \) mutants, especially in minimal medium with succinate (Table 1). Both operons are required for the biosynthesis of the \textit{Pseudomonas} quinolone signal (PQS) and 2-heptyl-4-hydroxyquinolone, which act as quorum sensing signal molecules together with the transcriptional regulator PqsR [15]. Transcript abundance of \( \text{pqzR} \) was also affected, but to a lesser extent (Table 1). As none of these genes showed elevated transcript levels in the \( \text{crc} \) mutant, their regulation by the \( \text{CbrAB/Crc} \) system is probably indirect. Moreover, several genes and operons involved in the anaerobic denitrification pathway were affected by the \( \text{CbrAB/Crc} \) system at the transcript level (Tables S1 and S2), although the cells had been grown aerobically. However, except for \textit{narG}, which has a poorly conserved CA motif (Table 1), none of the corresponding transcripts showed a CA motif, suggesting indirect regulation by \( \text{CbrAB/Crc} \). Finally, in cells cultivated in LB, \( \text{pqzA} \) (encoding the pyrroloquinoline quinone [PQQ] biosynthesis protein A) was one of the most strongly up-regulated genes regulated by \( \text{CbrAB/Crc} \) (Table 1). As none of these genes showed elevated transcript levels in the \( \text{crc} \) \( \text{Z} \) mutant, their regulation by the \( \text{CbrAB/Crc} \) system is probably indirect. Moreover, several genes and operons involved in the anaerobic denitrification pathway were affected by the \( \text{CbrAB/Crc} \) system at the transcript level (Tables S1 and S2), although the cells had been grown aerobically. However, except for \textit{narG}, which has a poorly conserved CA motif (Table 1), none of the corresponding transcripts showed a CA motif, suggesting indirect regulation by \( \text{CbrAB/Crc} \).

Validation of candidate targets - In the remainder of this study, we will focus on transcripts (\textit{estA}, \textit{acsA}, and \textit{aroP2}) that have increased abundance in a \( \text{crc} \) mutant and all contain a putative CA motif (i.e. AAnAAnAA). In addition, we investigated the role of the \( \text{CbrAB/Crc} \) system on \( \text{bkdR} \) and \( \text{bkdA1} \), as the \( \text{bkdA1} \) transcript was highly upregulated in the \( \text{crr} \) mutant and the cognate regulatory gene \( \text{bkdR} \) contains a putative CA-motif in the vicinity of its ribosome binding site. All these genes are involved in the utilization of less preferred carbon sources. Our initial concern was to validate the microarray results by an independent method. Data obtained by RT-qPCR (Table 2) were in full agreement with the microarray data (Table 1). In particular, the RT-qPCR measurements obtained in LB-grown cells proved to be more sensitive and hence possibly more accurate than the microarray data.

Regulation of uptake and degradation of poor carbon sources - Previously, only \textit{amiE} and \textit{phzM} mRNAs have been characterized as targets of the \( \text{CbrAB/Crc} \) system in \textit{P. aeruginosa} [1,18]. In this section, we will provide evidence that \textit{estA} and \textit{acsA} are also targets. EstA esterase is an autotransporter that possesses lipolytic enzyme activity [19] and contributes to the hydrolysis of glycerol esters with short-chain fatty acids, which can be further used as carbon sources by \textit{P. aeruginosa} [20]. EstA is located in the outer membrane [21]. In our RT-qPCR analysis, \textit{estA} mRNA appeared to be down-regulated in \textit{PAO1}Δ\textit{crcZ} and \textit{PAO1}Δ\textit{cbrB} and up-regulated in \textit{PAO1}Δ\textit{crc} grown under both growth conditions (Table 2). A translational \textit{estA-lacZ} fusion was assayed for \( \beta \)-galactosidase activity in strain \textit{PAO1} and in the \( \text{crr} \), \( \text{crcZ} \) and \( \text{cbrB} \) mutants, after growth in LB (Figure 2A) and BSM supplemented with 40 mM

Figure 1. Venn diagrams summarizing changes in transcript abundance in mutants affected in the CbrAB/Crc cascade. Data are taken from Tables S1 and S2. Changes in transcript levels from LB vs. BSM-succinate cultures are shown for (A) \textit{PAO1}Δ\textit{crc}, (B) \textit{PAO1}Δ\textit{crcZ} and (C) \textit{PAO1}Δ\textit{cbrB}, by comparison with the wild-type strain. Overlaps between the differentially regulated transcripts in the \( \text{crc} \), \( \text{crcZ} \) and \( \text{cbrB} \) mutants are shown (D) for LB cultures and (E) for BSM-succinate cultures.

doi:10.1371/journal.pone.0044637.g001
| ORF* | Gene | LB<sup>b</sup> | BSM<sup>b</sup> + succinate | Description | CA motif (location)<sup>a</sup> |
|------|------|-------------|-----------------|-------------|------------------|
|      |      | crc vs. wt | cbr vs. wt      | crcZ vs. wt | crc vs. wt      | cbrB vs. wt | crcZ vs. wt |
| PA0755 | opdH | 5,9         | 2,05            |             | cis-aconitate porin OpdH | AAGAACA (−25 to −18) |
| PA0866 | aroP2 | 4,73        |                 |             | aromatic amino acid transport protein | AACAAUAA (−33 to −12) |
| PA0887 | acaA | −3,41       | −2,51           | −3,27       | 7,98            | 2,88        | 3,62        | acetyl-coenzyme A synthetase | AACAAAAACAA (−35 to −25) |
| PA0996 | pqiA | −3,55       | −3,49           | −2,01       | 18,34           | −16,67      |             | probable coenzyme A ligase |
| PA0997 | pqiB | −3,72       | −3,49           | −2,11       | 33,37           | −56,5       | PqiB protein |
| PA0998 | pqiC | −3,73       | −3,89           | −2,01       | 25,21           | −40,64      | PqiC protein |
| PA0999 | pqiD | −3,77       | −3,38           | −2         | 23,39           | −34,65      | 3-oxyacyl-(acyl-carrier-protein) synthase III |
| PA1000 | pqiE | −2,79       | −2,58           | −2,09       | 18,62           | −22,18      | quinolone signal response protein |
| PA1001 | phnA | −3,58       | −3,28           | −2,08       | 18,69           | −23,34      | anthranilate synthase component I |
| PA1002 | phnB | −2,04       | −2,01           | −11,69      | −11,46          | anthranilate synthase component II |
| PA1003 | pqrR | −2,48       |                 | −2,89       | 2,5             | 3,62        | HAI (MwFR) |
| PA1070 | braG | −2,28       | −3,76           | −3,22       | branched-chain amino acid transport protein | BraG |
| PA1071 | braF | −2,37       | −4,02           | 2,08        | −2,91           | branched-chain amino acid transport protein | BraF |
| PA1072 | braE | −2,21       | −3,11           | 2,33        | −3,05           | branched-chain amino acid transport protein | BraE |
| PA1073 | braD | −2,88       | 2,5             | −5,56       | branched-chain amino acid transport protein | BraD |
| PA1074 | braC | −2,11       | −3,6            | 2,14        | −2,41           | branched-chain amino acid transport protein | BraC |
| PA1342 | −2,68 | −2,38       |                 | probable binding protein component of ABC transporter | AAUAAAAA (−33 to −26) |
| PA1617 | 2,44 |             |                 | probable AMP-binding enzyme | AACACAAACAA (−23 to −13) |
| PA1894 | 5,83 | −4,24       |             | hypothetical protein |
| PA1895 | 4,27 | −3,07       |             | hypothetical protein |
| PA1984 | exaC | −2,76       | 3,84           | −3,37       | NAD+ dependent aldehyde dehydrogenase | ExaC |
| PA1985 | pqqA | 11,34       | 8,74           |             | pyrroloquinoline quinone biosynthesis protein A |
| PA1986 | pqqB | 2,47        | 2,52           |             | pyrroloquinoline quinone biosynthesis protein B |
| PA1987 | pqqC | 3,86        | 3,7            |             | pyrroloquinoline quinone biosynthesis protein C |
| PA1988 | pqqD | 3,7         | 4,02           |             | pyrroloquinoline quinone biosynthesis protein D |
| PA1989 | pqqE | 2,72        | 2,77           |             | pyrroloquinoline quinone biosynthesis protein E |
| PA2247 | bkdA1 | −2,36       | −3,81           | 3,23        | 2-oxoisovalerate dehydrogenase (alpha subunit) |
| PA2248 | bkdA2 | −2,6       | −3,51           |             | 2-oxoisovalerate dehydrogenase (beta subunit) |
| PA2249 | bkdB | −2,48       | −3,22           |             | branched-chain alpha-keto acid dehydrogenase |
| PA2250 | lpdV | −2,62       | −3,07           |             | lipoamide dehydrogenase-Val |
| PA2533 | 2,13 |             |                 | probable sodiumalanine symporter | AACAAAGAAUA (−20 to −10) |
| PA3038 | −4,13 | 16,75      | 2,11           | probable porin | AAUAAAC (−7 to +1) |
| PA3190 | −7,43 | −7,81       |                 | probable binding protein component of ABC sugar transporter | AAUAAAC (−24 to −17) |
| PA3366 | amiE | 4,65        | 2,04           |             | aliphatic amidase | AACACAA (−20 to −13) |
| PA3452 | mqaA | 4,87        | 3,12           |             | malate:quinone oxidoreductase |
| PA3570 | mmsA | 2,23        |                 |             | methylmalonate-semialdehyde dehydrogenase | AACAAUAA (−37 to −30) |
| PA3875 | narG | −6,01       | −4,46           | −4,79       | respiratory nitrate reductase alpha chain | AAGAAGAA (+34 to +41) |
| PA4139 | −3,37 | 2,87        | 4,72           |             | hypothetical protein |
| PA4147 | acor | 3,38        |                 |             | transcriptional regulator Acor | AACAAAC (−30 to −23) |
| PA4150 | acoA | 2,77        |                 |             | probable dehydrogenase E1 component | AACAAAC (−9 to −2) |
| PA4151 | acoB | 4,1         |                 |             | acetoin catabolism protein AcoB | AACAAAC (−22 to −15) |
| PA4306 | flip | −2,9        | −6,66           | −8,64       | Type IVb pilin, Flp | AACAAAC (−22 to −15) |

**Table 1.** Selection of targets of the CbrAB/Crc system implicated by the transcriptome analysis.

<sup>**a**</sup> Motif localization is described as the location of the 5′-end matching of the motif within the coding region of the ORF.

<sup>**b**</sup> LB and BSM refer to LB and BSM media conditions, respectively.
succinate as the sole carbon source (Figure 2B). Under both conditions, estA-lacZ expression was repressed in PAO1 ΔcbrB and derepressed in PAO1 ΔcbrZ. However, the estA-lacZ expression profiles showed quantitative differences in LB (Figure 2A) vs. BSM-succinate (Figure 2B), in agreement with the transcript analysis (Tables 1 and 2). To confirm that estA is a target of the CbrAB/Crc system we mutated the CA motif, which is located 17 nucleotides upstream of the estA start codon (Table 1). In the absence of the wild-type CA motif, the CbrAB/Crc system lost its ability to regulate estA completely (Figure 2C).

P. aeruginosa can use acetate as a sole carbon and energy source, by converting acetate to acetyl-CoA, which is further metabolized via the tricarboxylic acid and glyoxyllic acid cycles to generate energy and cell components. The initial reaction is catalyzed by acetyl-CoA synthetase, the product of the acsA gene. Acetyl-CoA synthetase activity is induced during growth on acetate or on ethanol [16, 22]. In our transcript analysis, acsA was differentially expressed in the wild type PAO1 and the cbrB and cbrZ mutants (Tables 1 and 2). The unexpected and unexplained finding here is that the cbrB and cbrZ mutants on the one hand and the ccr mutant on the other did not show opposite effects on acsA expression (Table 1). To demonstrate that the CbrAB/Crc system regulates acsA, we measured the β-galactosidase activity of a translational acsA-lacZ fusion in these strains. As strain PAO1 lacking ccrZ or cbrB showed a severe growth defect on acetate as the sole carbon source (Figure 3A), we grew the cells in BSM supplemented with 40 mM acetate and 5 mM succinate. As expected, acsA-lacZ expression was completely repressed in PAO1 Δacsc and PAO1 ΔcbrB, and increased in PAO1 ΔcbrZ, by comparison with PAO1 (Figure 3B). The acsA mRNA harbours an extended CA motif (AACAAAAAAGA) located 25 nucleotides upstream of the acsA start codon. Mutation of the CA motif in the acsA leader of an

### Table 2. Fold changes of transcript abundance in Δcbr, ΔcbrZ and ΔcbrB mutants compared to wild type PAO1 in LB and BSM + succinate as determined by RT-qPCR.

| Gene | LB<sup>a</sup> | BSM<sup>b</sup> + succinate |
|------|----------------|-----------------------------|
|      | crc vs. wt     | crcZ vs. wt                 | cbr vs. wt | cbrZ vs. wt |
| estA | 2.6 ± 0.4      | 2.5 ± 0.4                   | 2.6 ± 0.4  | 2.5 ± 0.4 |
| acsA | 4.3 ± 0.2      | 7.5 ± 0.2                   | 7.5 ± 0.2  | 7.5 ± 0.2 |
| bkdR | 2.3 ± 0.4      | 1.4 ± 0.2                   | 2.3 ± 0.4  | 1.4 ± 0.2 |
| bkdA1| 4.0 ± 0.2      | 9.3 ± 0.1                   | 9.3 ± 0.1  | 9.3 ± 0.1 |
| ilvE | 3.2 ± 0.2      | 1.2 ± 0.2                   | 1.2 ± 0.2  | 1.2 ± 0.2 |
| groP2| 2.7 ± 0.4      | 1.4 ± 0.1                   | 1.4 ± 0.1  | 1.4 ± 0.1 |

<sup>a</sup>Values are represented as averages of 3 independent replicates for every strain.

<sup>b</sup>doi:10.1371/journal.pone.0044637.t001
Figure 2. EstA esterase is a target of the CbrAB/Crc cascade. β-Galactosidase activities of an estA’-'lacZ fusion were measured in PAO1 (black diamonds), PAO1ΔcbrB (black triangles), PAO1ΔcrcZ (black squares) and PAO1Δcrc (black circles) harbouring a plasmid with a translational estA’-'lacZ fusion (pTLestA) in LB medium and (B) in BSM medium supplemented with 40 mM succinate. (C) β-Galactosidase activities derived from a translational estA’-'lacZ fusion with a mutated CA motif (pTLestA-ΔCA) were determined in PAO1 (black diamonds), PAO1ΔcbrB (black triangles), PAO1ΔcrcZ (black squares) and PAO1Δcrc (black circles). The strains were grown in BSM supplemented with 40 mM succinate. Cell growth was monitored by measuring the optical density at 600 nm (OD600) (white symbols). doi:10.1371/journal.pone.0044637.g002

Figure 3. Growth on acetate and acsA expression are under CbrAB/Crc control. (A) Growth of PAO1 (black diamonds), PAO1ΔcbrB (black triangles), PAO1ΔcrcZ (white squares) and PAO1Δcrc (white circles) was measured in BSM supplemented with 40 mM acetate. (B) β-Galactosidase expression of a translational acsA’-'lacZ fusion (pME10044) was monitored in PAO1 (black diamonds), PAO1ΔcbrB (black triangles), PAO1ΔcrcZ (black squares) and PAO1Δcrc (black circles). (C) Expression of a translational acsA’-'lacZ fusion where the CA motif had been mutated (pME10045) was followed in PAO1 (black diamonds) and PAO1Δcrc (black circles). Cells were grown in BSM supplemented with 5 mM succinate and 40 mM acetate. The corresponding growth curves are shown in white symbols. doi:10.1371/journal.pone.0044637.g003
Figure 4. Utilization of branched-chain amino acids is under CbrAB/Crc control. (A) Growth of PAO1 (black diamonds), PAO1ΔcbrB (black triangles), PAO1ΔcrcZ (white squares) and PAO1Δcrc (black circles) was monitored in BSM supplemented with 23 mM leucine, 25.6 mM valine and 7.6 mM isoleucine as the sole carbon sources. β-Galactosidase activities conferred by (B) a translational bkdA1′-lacZ fusion (pME10049) and (C) a translational bkdR′-lacZ fusion (pME10048) were measured in PAO1 (black diamonds), PAO1ΔcbrB (black triangles), PAO1ΔcrcZ (black squares) and PAO1Δcrc (black circles). Cells were grown in LB supplemented with 23 mM leucine, 25.6 mM valine and 7.6 mM isoleucine. The corresponding growth curves are shown in white symbols. doi:10.1371/journal.pone.0044637.g004

Figure 5. Tyrosine uptake is under CbrAB/Crc control. (A) Growth of PAO1 (black diamonds), PAO1ΔcbrB (black triangles), PAO1ΔcrcZ (white squares) and PAO1Δcrc (white circles) was followed in BSM supplemented with 4 mM tyrosine as the sole carbon source. (B) β-Galactosidase activities conferred by the translational aroP2′-lacZ fusion (pME10046) were determined in PAO1 (black diamonds), PAO1ΔcbrB (black triangles), PAO1ΔcrcZ (black squares) and PAO1Δcrc (black circles). (C) Expression of a translational aroP2′-lacZ fusion with a mutated CA motif (pME10047) was similarly followed in PAO1 (black diamonds) and PAO1Δcrc (black circles). Cells were grown in synthetic sputum medium (SCFM). The corresponding growth curves are shown in white symbols. doi:10.1371/journal.pone.0044637.g005
acmA'-lacZ construct resulted in loss of catabolite repression mediated by Crc (Figure 3C).

**Targets of the CbrAB/Crc system in the uptake and metabolism of amino acids** - The CbrAB two-component system is important for maintaining the C to N balance during amino acid utilization by *P. aeruginosa* [23]. In a seminal study, Crc was found to be a post-transcriptional regulator of the bkd operon, which is involved in the utilization of branched-chain amino acids in *P. putida* and *P. aeruginosa* [24]. Furthermore, a recent proteomic analysis [7] revealed that Crc is involved in the regulation of several transporters required for the uptake of amino acids in *P. aeruginosa*. Our transcriptome analysis revealed a number of CbrAB/Crc-regulated genes and operons (e.g., bkdA1A2B-ldv, braBCDEFG, arcP2, araBEFG, araP, arcBC) that are involved in the utilization and transport of amino acids. In agreement with the original observations of Hester et al. [24], we found that the CbrAB/Crc system is involved in the regulation of branched-chain amino acid utilization in *P. aeruginosa*. In BSM supplemented with valine, isoleucine and leucine as the sole carbon sources, strains PAO1ΔcrcZ and PAO1ΔacbB were not able to grow, whereas strains PAO1 and PAO1Δacr did grow (Figure 4A). Branched-chain amino acid assimilation requires an initial deamination step performed by the branched-chain amino acid transaminase (encoded by ibcE), followed by decarboxylation by the branched-chain keto acid dehydrogenase (encoded by the bkdA1A2B-ldv operon). The bkd operon is induced by branched-chain amino acids as well as branched-chain keto acids [25]. In *P. putida*, all five genes are repressed by Crc [26], whereas in *P. aeruginosa* the only the bkd operon, but not the ibcE gene, seems to be under catabolite repression control by Crc (Tables 1 and 2). To verify the results from our transcriptome data, we analyzed the expression of a translational bkdA1'-lacZ fusion in LB supplemented with 23 mM leucine, 25.6 mM valine and 7.6 mM isoleucine. As shown in Figure 4B, bkdA1'-lacZ expression was slightly increased in PAO1Δacr in comparison with that in PAO1, and strongly repressed in PAO1ΔarcZ and PAO1ΔcbrB. In *P. putida*, a CA motif (AAAAACAAA) is located 24 nucleotides upstream of the bkdA1 start codon. By contrast, in *P. aeruginosa* a putative CA motif (AAAAACAAAA) occurs 187 nucleotides upstream of the bkdA1 start codon and it is questionable whether this sequence would affect translation initiation of bkdA1. A more likely scenario pictures the bkdR gene, which lies upstream of the bkd operon, as a target of the CbrAB/Crc system. In *P. putida*, the transcriptional regulator BkdR positively controls expression of the bkd operon [27]. The BkdR protein content was elevated in a crc mutant while the bkdR mRNA levels were unchanged, whereas bkdA1 expression was affected at both the mRNA and the protein level [28].

In *P. aeruginosa*, two putative CA motifs can be found in the untranslated leader region of bkdR (AACUACAGAAA at −26 to −20 and AACAGAGAAAAC at −30 to −7). To see whether the CbrAB/Crc system regulates the expression of bkdR, we performed β-galactosidase assays of a translational bkdR'-'lacZ fusion in LB supplemented with 23 mM leucine, 25.6 mM valine and 7.6 mM isoleucine. The activity of this fusion was 1.5- to 2.0-fold increased in PAO1Δacr compared to PAO1 and was practically undetectable in PAO1ΔarcZ and PAO1ΔacbB (Figure 4C), confirming that bkdR is a target of the CbrAB/Crc system in *P. aeruginosa*.

Another good candidate for interaction with the CbrAB/Crc system was the arcP2 gene, which encodes a transporter for aromatic amino acids. A growth experiment was performed with PAO1 and itscrcZ, cbrB, andacbB deletion mutants in BSM supplemented with 4 mM tyrosine as the sole carbon source. ThecrcZ and cbrB mutants did not grow, whereas PAO1 and PAO1Δarc reached an OD_{600} of 1 after 9 h; PAO1Δarc even grew slightly faster than the wild type strain (Figure 5A). The expression of a translational arcP2'-lac fusion was very low in LB and in BSM amended with tyrosine (data not shown), as would be expected for a gene specifying an inner membrane protein. It has been shown previously that arcP2 expression is up-regulated in a lyophilized CF sputum medium, which is rich in amino acids [29]. Therefore, we used a synthetic CF sputum medium (SCFM; [30]) for measuring the β-galactosidase activities conferred by the arcP2'-lac fusion. Although expression was still very low under these conditions, a 2- to 4-fold higher expression of the arcP2'-lac fusion was measured in PAO1Δacr, compared with PAO1; in the crcZ and cbrB mutants arcP2 was fully repressed (Figure 5B). The deletion of the CA motif in the fusion construct resulted in loss of regulation by Crc (Figure 5C) and confirmed that arcP2 is a target of the CbrAB/Crc system.

**Consequences of catabolite repression for environmental adaptation of P. aeruginosa** - As we have shown that the CbrAB/Crc system strongly affects the utilization and transport of various less preferred carbon and nitrogen sources, the question arises as to what benefits *P. aeruginosa* may obtain from this regulation in natural environments, e.g. in the CF lung where amino acids are important C sources [29,30]. To investigate the impact of catabolite repression under such conditions, we cultivated PAO1, PAO1Δacr, PAO1ΔarcZ and PAO1ΔacbB aerobically in the artificial sputum medium (SCFM). ThecrcZ and cbrB deletion mutants grew more slowly than the PAO1 wild type, whereas the crc deletion strain grew similarly to PAO1 (Figure 6). In this sputum medium, tyrosine and phenylalanine are important C sources and degradation of both amino acids is induced in *P. aeruginosa* [31]. Moreover, leucine (1.6 mM), isoleucine (1.1 mM) and valine (1.1 mM) are present at high concentrations in this medium [30]. As we have shown above, tyrosine uptake (Figure 4) and utilization of branched-chain amino acids (Figure 5) are tightly controlled by the CbrAB/Crc system. The growth patterns seen in SCFM (Figure 6) are fully consistent with this regulation. As during chronic infection *P. aeruginosa* forms biofilms [32], we also measured biofilm formation in PAO1, PAO1Δacr, PAO1ΔarcZ and PAO1ΔacbB in SCFM using a static biofilm assay. ThecrcZ and cbrB mutants were impaired in biofilm formation whereas the biofilm formed by PAO1Δacr was increased ~5-fold, by compar-

![Figure 6. Growth of P. aeruginosa in sputum medium is under CbrAB/Crc control.](image-url) Growth curves of PAO1 (black diamonds), PAO1ΔcbB (black triangles), PAO1ΔcrcZ (white squares) and PAO1Δarc (white circles) were obtained in SCFM. doi:10.1371/journal.pone.0044637.g006
Discussion

In our present transcriptome analysis, the expression of \( \geq 380 \) genes was affected by mutations in the CbrAB/Crc system in \textit{P. aeruginosa} when we take into account data obtained in LB and in the defined medium BSM-succinate. Thus, in spite of a major output at the level of translation, the CbrAB/Crc signal transduction pathway clearly has a global impact on transcript abundance. In agreement with earlier phenotypic data [1], the present transcriptomic data indicate that most CbrB activity is linked to CrcZ activity (Figure 1) in that transcriptomic responses were similar, but not identical in cbrB and crcZ mutants (Tables S1 and S2). It is therefore possible that CbrB could act as a transcriptional regulator of genes other than the previously recognized \textit{crc} \( \rightarrow \) \textit{lip} \( \rightarrow \) and \textit{hatU} genes [5]. However, such novel CbrB target genes remain to be identified. Whether CrcZ regulates mRNA expression independently of Crc is another question that cannot be answered by the experiments of this study.

The overlap between Crc- and CrcZ-regulated transcripts was unexpectedly low (Figure 1). There may be several reasons for this finding. In general, mRNAs are more stable when they are translated [33]. Nevertheless, in a \textit{crc} mutant, derepressed translation may not always result in more stable, hence more abundant transcripts, whereas in a \textit{crcZ} mutant, in which translational initiation of target mRNAs is repressed, the (negative) effects on transcript abundance may be more obvious. However, under both growth conditions used here, expression of \textit{crcZ} is low and thus Crc-mediated repression of target mRNAs by Crc is strong [1]. Therefore, a \textit{crcZ} mutation may result only in minor and possibly undetectable changes of the expression of some genes. As shown in Table 2, RT-qPCR (as a more sensitive technique than the microarray analysis) established that the \\textit{cck, accA, bkdA1} and \textit{aroP2} transcripts were regulated by Crc and CrcZ/CbrB in an opposite manner. However, there are also limitations. For example, \textit{bkdR} did not reveal changes of transcript levels, whereas a \textit{bkdR\rightarrow lacZ} reporter fusion was regulated (Table 2). Finally, many genes were not induced at the transcriptional level under the growth conditions used, making changes due to catabolite repression difficult to detect. Despite such limitations, we have been able to use transcriptome analysis as a tool to identify several novel targets of the CbrAB/Crc system.

The main targets of the CbrAB/Crc system seem to be metabolic transcripts, which is expected for a catabolite repression control system. Moreover, as noted before [6,7,34], the CbrAB/Crc system not only regulates genes for the utilization of less preferred C sources, but also dramatically influences motility and biofilm development, traits that are involved in the pathogenicity of \textit{P. aeruginosa} and that depend on nutritional conditions. For example, a defect in CbrAB-mediated regulation of aromatic and branched-chain amino acid utilization affected growth and biofilm development of \textit{P. aeruginosa} in sputum medium (Figures 6 and S2). In addition to \textit{aroP2} and \textit{bkdR} (Figures 4 and 5), the \textit{braC} gene and the \textit{braDEFG} operon (encoding transport proteins with high affinity for the branched-chain amino acid leucine, isoleucine and valine and with low affinity for alanine and threonine; [35,36]), were down-regulated in the \textit{crcZ} mutant in both media and up-regulated in the \textit{crc} mutant of \textit{P. aeruginosa} (Table 1). An incomplete CA motif (UACAACAA at +23 to +36) in the coding region of \textit{braD} as well as an extended, but imperfect CA motif (ACAACAAUGA-CAACA at -13 to -6) upstream of the \textit{braC} start codon suggests that the CbrAB/Crc system also controls these genes. However, further investigation is required to confirm this regulation.

In sputum medium a \textit{crc} mutant had no growth advantage over the wild type (Figure 6) and there is currently no evidence that colonization of the CF lung by \textit{P. aeruginosa} would select for \textit{crc} mutants. However, both the \textit{crcZ} and the \textit{cbrB} mutant were partially deficient for growth in SCFM and may therefore be affected in virulence.

It has been known for some time that nutritional conditions influence biofilm development [37,38] and that the CbrAB/Crc system is an important regulator for biofilm formation, although some published results appear to be strain-specific [6,7,34,39]. For example, a PA14 \textit{Δcrc} mutant produced less biofilm in minimal media containing glucose [6,34] whereas a PA01 \textit{Δcrc} strain showed enhanced biofilm formation in LB compared with the wild type strain [7]. Our data indicate that the impact of the CbrAB/Crc system on biofilm development depends on media and carbon sources (Figure S2). In BSM medium, different carbon sources clearly affected the formation of static biofilms (Figure S2), which is consistent with different carbon sources having different regulatory effects on targets of the CbrAB/Crc system. By contrast, in LB medium the typical pattern of upregulation in the \textit{crc} mutant and strong repression in the \textit{crcZ} and \textit{cbrB} deletion strains was not observed. In conclusion, the CbrAB/Crc system acts on a variety of different transcripts involved in biofilm development. Finding out more about the relationships between nutrient availability, catabolite repression control and regulation of virulence and biofilm development in \textit{P. aeruginosa} may ultimately lead to a better understanding of the bacterium’s pathogenicity and intrinsic resistance to antimicrobial agents.

Methods

\textbf{Bacterial strains and growth conditions -} The strains and plasmids used in this study are listed in Table S3. Unless indicated otherwise, cells were grown in Luria Broth (LB) [12,40] or in a minimal medium (BSM) [41] supplemented with 40 mM succinate [1]. For the investigation of special targets and growth behavior we used BSM amended with either 4 mM tyrosine, 5 mM succinate +40 mM acetate, 40 mM acetate or 23 mM leucine +25.6 mM valine +7.6 mM isoleucine as the sole carbon sources and LB supplemented with 23 mM leucine +25.6 mM valine +7.6 mM isoleucine. The synthetic sputum medium SCFM has been described by Palmer et al. [30]. When required, antibiotics were added to the media at the following concentrations: 100 µg ml\(^{-1}\) ampicillin for \textit{Escherichia coli} and 125 µg ml\(^{-1}\) tetracycline for \textit{P. aeruginosa}.

\textbf{Transcriptome analysis -} Overnight cultures of \textit{P. aeruginosa} PAO1, the \textit{ΔcbrB} mutant PAO6711, the \textit{Δcrc} mutant PAO6673 and the \textit{ΔcrcZ} mutant PAO6679 were diluted to an initial OD\(_{600}\) of 0.05 in 20 ml of LB or BSM amended with 40 mM succinate. The cultures were grown at 37°C with vigorous shaking until they reached an OD\(_{600}\) of 1.6. RNA purification, cDNA synthesis and cDNA hybridization were performed as described previously [42]. Processing of the \textit{P. aeruginosa} GeneChip Array (Affymetrix) was performed at the University of Lausanne Center for Integrative Genomics. For each condition, cultures were grown in triplicate, and RNAs from these cultures were pooled before proceeding to...
cDNA synthesis. In addition, one biological replicate for each condition was performed on a separate day and run on separate microarray chips. Transcripts tabulated in Tables S1 and S2 meet the following criteria: (i) the P-value for each transcript analyzed is ≤0.05 and (ii) the change in transcript level is ≥2.0-fold. In compliance to MIAME guidelines, the data has been deposited in the GEO database, GEO accession number: GSE33243.

Real-time quantitative PCR (RT-qPCR) – PA01, PA01Δcrbc and PA01Δcrbc mutant PA06711, PA01Δcrbc mutant PA06673 and PA01Δcrbc mutant PA06679 were grown in LB medium or BSM supplemented with 40 mM succinate at 37°C with vigorous shaking until they reached an OD600 of 1.6. Cells were harvested using the RNA bacteria protect solution (QIAGEN). Total RNA was extracted with the RNA RNaseeasy kit (QIAGEN), treated with RQ1 DNase (Promega) to remove contaminating genomic DNA and subsequently re-purified using phenol-chlorophorm extraction. cDNA from each sample was obtained as previously described [16] for every sample 500 ng of total RNA was used. The resulting cDNA were used as templates for qPCR and quantitated with a Bio-Rad Icyycler machine using a Sybr Green Quantitect kit (QIAGEN). Fold changes were estimated by a comparative threshold cycle method with the ∧∧ct gene PA1544 as a standard [43]. The primer pairs, termed gene name fw and gene name rev, used for qRT-PCR are shown in Table S4.

Construction of plasmids – To construct translational lacZ fusions to estA, bkdA and bkdR, fragments of 592 bp, 354 bp and 368 bp, respectively, were amplified by PCR with the primer pairs Q67_estAfw/R67_estArev (estA), PA2246fw/PA2246rev (bkda) and PA2247fw/PA2247rev (bkdr) (Table S4) and chromosomal DNA of strain PA01 as template. The fragments containing the respective authentic promoter regions and the translation initiation sites were fused in-frame after the 6th codon of estA and the 4th codon of the bkda and bkdr genes to the 8th codon of lacZ. These fragments were digested with EcoRI and BamHI and cloned into the corresponding sites of pME6015 generating pTL_estA (estA-lacZ), pME10049 (bkda1-lacZ) and pME10048 (bkdr1-lacZ). The translational accA-lacZ and arsP2-lacZ fusions were constructed as described above by amplifying a 202-bp and 316-bp PCR fragment with primers AccsA/AcsA2 and ArpP21/AroP22 (Table S4), respectively. These fragments were digested with EcoRI and PstI and cloned into the corresponding sites of pME6015, resulting in plasmid pME10044 (accA-lacZ) and pME10046 (arsP2-lacZ).

The CA motif mutations (TCAGTAGC instead of AAAAAAACAA of same procedure was used to mutate the CA motif sequences (Table S4), respectively. The resulting primers were named pME10045 (accA-ACA) and pME10047 (arsP2-ΔCA). All mutations were confirmed by sequencing.

β-Galactosidase assays – β-Galactosidase activities were quantified by the Miller method [40], using cells permeabilized with 5% toluene or 5% (v/v) chloroform and 0.01% SDS. Values shown were derived from three independent experiments and are means ± standard deviation.

Static biofilm assay – A static-culture biofilm assay in microtiter plates was performed as described previously [44]. Cells were grown in LB, SCFM or BSM medium supplemented with 40 mM succinate, glucose or mannitol, respectively, for 24 h and biofilms were stained with 0.1% crystal violet solution. The dye bound, which is proportional to the biofilm produced, was solubilized with 95% (v/v) ethanol and after stripping with 96% (v/v) ethanol the absorption was photometrically measured at 540 nm (A540).

Supporting Information

Table S1 Transcripts which were at least two-fold differentially expressed in PA01Δcrcc in compared to PA01.

Table S2 Transcripts which were differentially regulated in the cbrB and crcZ mutants compared to PA01.

Table S3 Strains and plasmids used in this study.

Table S4 DNA oligonucleotides used in this study.

Acknowledgments

We thank Otto Hagenbächle, Mélanie Dupasquier and Sylvain Prader- vand for performing the microarray analyses and Karl Perron and Verena Ducret for help with the RT-qPCR experiments.

Author Contributions

Conceived and designed the experiments: ES MV NW FZH DH KL. Performed the experiments: ES MV NW FZH KL. Analyzed the data: ES MV NW FZH DH KL. Wrote the paper: ES DH KL.

References

1. Sonneleitner E, Abdou L, Haas D (2009) Small RNA as global regulator of carbon catabolite repression in Pseudomonas aeruginosa. Proc Natl Acad Sci USA 106: 21066–21071.

2. Moreno R, Marzi S, Rojo F (2009) The Crc global regulator binds to an unpaired Arich motif at the Pseudomonas putida alaS mRNA coding sequence and inhibits translation initiation. Nucleic Acids Res 37: 7678–7690.

3. Rojo F (2010) Carbon catabolite repression in Pseudomonas: optimizing metabolic versatility and interactions with the environment. EMBO Microb Rev 34: 658–684.

4. Sonneleitner E, Haas D (2011) Small RNAs as regulators of primary and secondary metabolism in Pseudomonas species. Appl Microbiol Biotechnol 91: 63–79.

5. Abdou L, Chou H-T, Haas D, Lu C-D (2011) Promoter recognition and activation by the global response regulator CbrB in Pseudomonas aeruginosa. J Bacteriol 193: 2784–2792.
25. Martin RR, Marshall VD, Sokatch JR, Unger L (1973) Common enzymes of P. aeruginosa. J Bacteriol 115: 190–204.

26. Moreno R, Martinez-Gonzariz M, Yuile L, Gil C, Rojo F (2009) The Pseudomonas putida Crc global regulator controls the hierarchical assimilation of amino acids in a complete medium: evidence from proteomic and genomic analyses. Proteomics 9: 2910–2926.

27. Madhu Sudan KT, Lorenz D, Sokatch JR (1993) The bkdR gene of Pseudomonas aeruginosa is required for expression of the bkd operon and encodes a protein related to Lrp of Escherichia coli. J Bacteriol 175: 3934–3940.

28. Hester KL, Madhu Sudan KT, Sokatch JR (2000) Catabolite repression control by crc in 2oxT medium is mediated by posttranscriptional regulation of bkdR expression in Pseudomonas putida. J Bacteriol 182: 1150–1153.

29. Palmer KL, Mashburn LM, Singh PK, Whiteley M (2005) Cystic fibrosis spum supports growth and cys key aspects of Pseudomonas aeruginosa physiology. J Bacteriol 187: 5267–5277.

30. Palmer KL, Aye LM, Whiteley M (2007) Nutritional cues control Pseudomonas aeruginosa multicellular behavior in cystic fibrosis spum. J Bacteriol 189: 8079–8087.

31. Palmer GC, Palmer KC, Joth PH, Whiteley M (2010) Characterization of the Pseudomonas aeruginosa transcriptional response to phenolalanine and tyrosine. J Bacteriol 192: 2722–2728.

32. de Keost TR (2009) Quorum sensing in Pseudomonas aeruginosa biofilms. Environ Microbiol 11: 279–288.

33. Kaberdin VR, Blasi U (2006) Translation initiation and the fate of bacterial mRNAs. FEMS Microbiol Rev 30: 967–979.

34. O'Toole GA, Gilsb KA, Hager PV, Phibbs PV Jr, Kolter R (2000) The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by Pseudomonas aeruginosa. J Bacteriol 182: 425–431.

35. Hoshino T, Kageyama M (1980) Purification and properties of a binding protein for branched-chain amino acids in Pseudomonas aeruginosa. J Bacteriol 141: 1055–1063.

36. Hoshino T, Kose K (1990) Cloning, nucleotide sequences, and identification of the products of the Pseudomonas aeruginosa PAO genes, which encode the high-affinity branched-chain amino acid transport system. J Bacteriol 172: 5531–5539.

37. Costerton JW, Levandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial biofilms. Annu Rev Microbiol 49: 711–745.

38. O'Toole GA, Kolter R (1998) Initiation of biofilm formation by Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 28: 449–461.

39. Amaral CI, Canosa I, Govantes F, Santero E (2010) Lack of CbrB in Pseudomonas aeruginosa affects not only amino acids metabolism but also different stress responses and biofilm development. Environ Microbiol 12: 1748–1761.

40. Miller LH (1972) Experiments in Molecular Genetics. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

41. Durham DR, Phibbs PV Jr (1982) Fractionation and characterization of the phosphoethanolmonovatate: fructose 1-phosphofructokinase system from Pseudomonas aeruginosa. J Bacteriol 149: 534–541.

42. Sonnleitner E, Gonzalez N, Sorger-Domenigg T, Heeb S, Richter AS, et al. (2011) The small RNA PhrS stimulates synthesis of the Pseudomonas aeruginosa quinolone signal. Mol Microbiol 80: 868–885.

43. Caille O, Rosier C, Perron K (2007) A copper-activated two-component system interacts with zinc and imipenem resistance in Pseudomonas aeruginosa. J Bacteriol 189: 4561–4566.

44. Merrit JH, Kadouri DE, O'Toole GA (2005) Growing and analyzing static biofilms. Current Protocols in Microbiology 1B.1–1B.1.17.