Hierarchical management of carbon sources is regulated similarly by the CbrA/B systems in *Pseudomonas aeruginosa* and *Pseudomonas putida*

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The CbrA/B system in pseudomonads is involved in the utilization of carbon sources and carbon catabolite repression (CCR) through the activation of the small RNAs *crcZ* in *Pseudomonas aeruginosa*, and *crcZ* and *crcY* in *Pseudomonas putida*. Interestingly, previous works reported that the CbrA/B system activity in *P. aeruginosa* PAO1 and *P. putida* KT2442 responded differently to the presence of different carbon sources, thus raising the question of the exact nature of the signal(s) detected by CbrA. Here, we demonstrated that the CbrA/B/CrcZ(Y) signal transduction pathway is similarly activated in the two *Pseudomonas* species. We show that the CbrA sensor kinase is fully interchangeable between the two species and, moreover, responds similarly to the presence of different carbon sources. In addition, a metabolomics analysis supported the hypothesis that CCR responds to the internal energy status of the cell, as the internal carbon/nitrogen ratio seems to determine CCR and non-CCR conditions. The strong difference found in the 2-oxoglutarate/glutamine ratio between CCR and non-CCR conditions points to the close relationship between carbon and nitrogen availability, or the relationship between the CbrA/B and NtrB/C systems, suggesting that both regulatory systems sense the same sort or interrelated signal.

**INTRODUCTION**

The genus *Pseudomonas* presents a great nutritional versatility and a rapid potential of adaptation to changing environmental conditions. The presence of a very high number of regulatory two-component systems (TCSs) encoded in their genomes has been postulated as a reason for such adaptability (dos Santos et al., 2004; Stover et al., 2000). In particular, the two specialized TCSs, CbrA/B and NtrB/C, are of major importance for *Pseudomonas* sensing and response to nutrient availability in its environment.

The CbrA/B system controls several catabolic pathways involving the utilization of various carbon and nitrogen sources, and modulates virulence and antibiotic resistance (Abdou et al., 2011; Amador et al., 2010; Itoh et al., 2007; Li & Lu, 2007; Nishijyo et al., 2001; Yeung et al., 2011; Zhang & Rainey, 2008). Indeed, mutations in the *cbrA* or *cbrB* genes render the bacterium incapable of growing on a variety of organic compounds as a sole carbon source, such as mannitol, glucose, pyruvate, citrate and some amino acids (e.g. arginine, histidine and proline), and a *cbrA* mutant strain exhibited increased resistance toward several clinical antibiotics (Nishijyo et al., 2001; Yeung et al., 2011). In addition, it was suggested that CbrA/B functions coordinately with NtrB/C to maintain a carbon/nitrogen balance (Amador et al., 2010; Li & Lu, 2007; Nishijyo et al., 2001).

Furthermore, CbrA/B is involved in carbon catabolite repression (CCR) control (Li & Lu, 2007; Nishijyo et al., 2001). CCR is a general mechanism by which the cell...
facilitates catabolism (assimilation) of carbon sources supporting efficient growth and represses catabolism of other carbon sources that are potentially usable, but less energetically favourable (reviewed by Görke & Stulke, 2008). In the presence of less-preferred substrates, the activity of the CbrA/B TCS is highly induced and the transcription of the non-coding small RNAs (sRNAs) crcZ in Pseudomonas aeruginosa, and crcZ and crcY in Pseudomonas putida increases (Abdou et al., 2011; García-Mauriño et al., 2013; Moreno et al., 2012; Sonnleitner et al., 2009). These sRNAs antagonize the activity of the Crc global regulator, leading to de-repression of the Crc mRNA targets involved not only in the catabolism of the bacterium, but also in its virulence, susceptibility to antibiotics and biofilm development (Linares et al., 2010; O’Toole et al., 2000).

In P. aeruginosa, CCR is known to be triggered by C4-dicarboxylates (including succinate, fumarate, malate and aspartate), i.e. preferred carbon sources (Collier et al., 1996; Liu, 1952; MacGregor et al., 1991; Valentini & Lapouge, 2013; Wolff et al., 1991). However, surprisingly, succinate has been described as a weak CCR inducer in P. putida (Hester et al., 2000a, b). Indeed, bkdR expression was demonstrated to be repressed by CCR, particularly by Crc, in both organisms (Hester et al., 2000b; Sonnleitner et al., 2012), where succinate inhibited the branched-chain keto acid dehydrogenase activity more strongly in P. aeruginosa than in P. putida (Hester et al., 2000b). The same concept was applicable to the amidase activity in P. aeruginosa and in P. putida, where the activity of the enzyme was higher in a medium containing succinate than in a medium containing glucose in P. putida (Hester et al., 2000b), whilst the opposite regulation was found in P. aeruginosa (Sonnleitner et al., 2009). Moreover, crcZ and crcY expression in P. putida was shown to be higher in a medium containing succinate than in a rich Luria–Bertani (LB) medium (Garcia-Mauriño et al., 2013; Moreno et al., 2012), whilst crcZ expression in P. aeruginosa was observed to be lower in a medium containing succinate than in a medium containing glucose or mannitol (Sonnleitner et al., 2009). As a consequence, the regulation of the benA gene by CCR in P. putida was investigated in a medium containing succinate as the non-CCR condition and rich LB medium as the CCR condition (Moreno et al., 2012), whereas the regulation of the amnE gene in P. aeruginosa was studied in succinate for CCR compared with glucose and then mannitol for non-CCR (Sonnleitner et al., 2009). It is noteworthy that Crc was shown to have the same function in both P. aeruginosa and P. putida as a P. aeruginosa crc mutant could be complemented with the heterologous crc of P. putida (Hester et al., 2000b).

In addition, the CbrA/B signalling transduction system was hypothesized not only to sense carbon sources, but also to sense the cellular carbon/nitrogen ratio maintaining a healthy balance between these two essential energy sources (Li & Lu, 2007; Nishiyio et al., 2001). Indeed, in P. putida, Crc activity was shown to be influenced by the carbon/nitrogen ratio used in the culture medium. When the carbon/nitrogen ratio was balanced, a Crc-dependent repression was observed for polyhydroxyalkanoate synthesis, whereas Crc-dependent repression was suppressed when the nitrogen source became limiting. Furthermore, this regulation was correlated with the levels of CrcZ/CrcY, being lower under the carbon/nitrogen balanced-ratio condition than under the nitrogen-limiting condition (La Rosa et al., 2014). Interestingly, it was also proposed that CCR could respond to the internal energy status of the cell rather than to external signals from the environment (Duetz et al., 1994, 1996; Rojo, 2010).

The previous reports suggested that the CbrA/B systems of P. aeruginosa PA01 and P. putida KT2442 are possibly activated by different CCR conditions and posed the question of the exact nature of the signal(s) detected by CbrA, which remains unknown to date. In our work, we reviewed these apparently contradictory results by a systematic comparison of the CbrA/B/CrcZ (Y) cascade in the two Pseudomonas species. For the first time, the same conditions of growth were adopted and the role of CbrA/B in activating crcZ expression was compared. We showed that the system was fully interchangeable in the two species and it responded similarly to the presence of different carbon sources. Furthermore, by adopting a metabolomics approach, we obtained data supporting the hypothesis that CCR responds to the internal energy status of the cell. Indeed, by comparing the metabolite profile of P. aeruginosa growing with a strong (fumarate) and weak [oxaloacetate (OAA)] CCR inducer, we could show that the internal carbon/nitrogen ratio may determine CCR and non-CCR conditions.

**METHODS**

**Bacterial strains and culture conditions.** The bacterial strains/plasmids and oligonucleotides used in this study are listed in Tables S1 and S2 (available in the online Supplementary Material), respectively. Strains were routinely grown in yeast broth or in basal salt medium (BSM) supplemented with different carbon sources and microelements as indicated in the figure legends (Amador et al., 2010; Reva et al., 2006; Kietisch et al., 2004; Sonnleitner et al., 2012; Yeung et al., 2011). Antibiotics were used at the following concentrations: 10 μg gentamicin ml⁻¹, 12.5 μg tetracycline ml⁻¹ for *Escherichia coli*; 50 μg gentamicin ml⁻¹, 125 μg tetracycline ml⁻¹ for *P. aeruginosa*; 10 μg gentamicin ml⁻¹, 25 μg ml⁻¹ tetracycline for *P. putida*. *P. aeruginosa* strains were grown at 37 °C and *P. putida* strains were grown at 30 °C.

**Construction of plasmids and gene replacement mutants.** DNA cloning and plasmid preparation were performed according to standard methods (Sambrook et al., 2000). Restriction and DNA-modifying enzymes were used following the instructions of the manufacturers. Electroporation was used for DNA transformation in *P. aeruginosa* and *P. putida* (Choi et al., 2006; Pessi & Haas, 2000).

The chromosomal *crcZ*-*pa–lacZ* fusion was constructed by ligating a 3.5 kb fragment, excised from plasmid pME9812 with EcoRI/XhoI and blunt-ended, into the mini-Tn7 vector pME7549 digested with *Stul*. The construct obtained (pME10046) and pUX-BF13 were introduced into recipient strains by co-electroporation, allowing transposition of the constructs into the chromosomal Tn7 attachment site of *P. aeruginosa* PA01 (Hummair et al., 2010; Zuber et al., 2003), leading to the strain PAO6816.
For the inactivation of the cbrA gene in P. aeruginosa PAO1, a 551 bp fragment containing the cbrA upstream region and the first 39 codons of cbrA and a 413 bp fragment containing the 79 last codons of cbrA and the downstream region were amplified by PCR using primer pairs cbrA1/cbrA2 and cbrA3/cbrA4, respectively. These products were digested with EcoRI/BgII and BglII/HindIII, and cloned into the EcoRI/HindIII sites in the suicide vector pME3087, yielding plasmid pME10104. Plasmid pME10104, carried by E. coli DH5α, was then introduced into P. aeruginosa PAO1 by triparental mating, using the helper strain E. coli HB101 (pRK2013). Merodiploids were resolved as described previously (Ye et al., 1995). The resulting strain, PAO6862, carried an in-frame cbrA deletion.

The mutant strain PAO6862 was complemented with the plasmid pME10105, carrying the cbrA gene, cbrA and its promoter region, 3595 bp, were amplified by PCR with the primer pair cbrAcompl1/cbrAcompl2 and subcloned into HindIII/SmaI sites of pME6182. The resulting plasmid pME10105 was then inserted into the chromosomal Tn7 attachment site (Humair et al., 2010; Zuber et al., 2003).

The ΔcbrA mutant in P. putida KT2442 was constructed as follows. Flanking regions of cbrA containing the upstream region to the first codon of cbrA (810 bp) and the coding region from codons 703 to 869 (469 bp) of cbrA were amplified by PCR using primer pairs ChrEF/Eco/ChrAR_Bam and DelPASRF2BamH/DelPASRRHind, respectively. Left and right fragments were digested with EcoRI/BamHI and BamHI/HindIII respectively, and the kanamycin resistance cassette, which was digested with BamHI from pMPO284, were cloned into the EcoRI/HindIII sites of plasmid pEX18Tc, resulting in plasmid pMPO1310. The construct obtained was introduced into the recipient strain by electroporation, and after the double event of recombination and excision of the cbrAcompl2 and subcloned into HindIII/SmaI sites of the pME6182. The resulting plasmid pMPO1317 was then introduced into the chromosomal Tn7 attachment site.

All plasmid constructions were verified by sequencing.

**GFP quantification.** Cultures of WT P. aeruginosa PAO1 and P. putida KT2442 strains were grown in 24-well plates each containing 1 ml BSM medium supplemented with a sole carbon source as listed in Fig. 1. Strains were carrying either the pME9824 (crcZpa-gfp) or the pPROBE-TT’ (empty) vector. Cell density (OD600) and GFP fluorescence (excitation 485BBI and emission EM520 filters) were measured with a Polar Star Omega microtitre plate fluorometer (BMG LABTECH). The fluorescence arbitrary units represent the fluorescence values corrected for the background (the WT strains carrying the empty vector).

**Metabolite extraction procedure.** Cultures of WT P. aeruginosa PAO1 were grown at 37 °C in 100 ml BSM supplemented with 40 mM OAA or 5 mM fumarate/40 mM OAA in 500 ml Erlenmeyer flasks with shaking at 180 r.p.m. and 37 °C. For each condition, two cultures were incubated in parallel. Samples were taken from each culture at various sampling times. Cells were filtered and metabolite extracted as described previously (Takeuchi et al., 2012).

**Capillary electrophoresis (CE)-time-of-flight (TOF)-MS.** CE-TOF-MS was carried out as described by Takeuchi et al. (2012) by Human Metabolome Technologies.

**β-Galactosidase assays.** β-Galactosidase assays were performed as described by Miller (1972) with bacterial cultures grown in BSM containing 40 mM fumarate or OAA as sole carbon source or rich LB medium. β-Galactosidase activity was measured in toluene-permeabilized cells and expressed in Miller units (Miller, 1972). P. aeruginosa or P. putida cells were grown in 100 ml Erlenmeyer flasks containing 20 ml medium or in 15 ml tubes containing 5 ml medium with shaking at 180 r.p.m. at 37 or 30 °C, respectively.

### RESULTS

**Effect of different carbon sources on crcZ expression in P. aeruginosa**

To better understand the activation of the CbrA/B/CrcZ (Y) signal transduction pathway in relation to Pseudomonas CCR, we decided to investigate the effect of various carbon sources on crcZ expression in P. aeruginosa and P. putida. Expression of the P. aeruginosa PAO1 crcZ-gfp reporter fusion was measured in (a) P. aeruginosa PAO1 and (b) P. putida KT2442 grown to exponential phase (OD600 ~0.3) in minimum medium containing different carbon sources or LB medium, as indicated. Values are given as percentage of expression in the OAA condition and are mean values of duplicate experiments with an SD of ± 10%.

![Fig. 1. crcZ induction is carbon source dependent in P. aeruginosa and P. putida. Expression of the P. aeruginosa PAO1 crcZ-gfp reporter fusion was measured in (a) P. aeruginosa PAO1 and (b) P. putida KT2442 grown to exponential phase (OD600 ~0.3) in minimum medium containing different carbon sources or LB medium, as indicated. Values are given as percentage of expression in the OAA condition and are mean values of duplicate experiments with an SD of ± 10%.](http://mic.sgmjournals.org/2245)
sources on the regulation of \(\text{crcZ}\) expression. This sRNA is the only one controlling CCR in \(P. \text{aeruginosa}\) and its expression was shown to be activated directly by the CbrA/B TCS (Abdou et al., 2011; Sonnleitner et al., 2009). Therefore, we reasoned that high expression levels of \(\text{crcZ}\) corresponded to high activity of the CbrA/B TCS and to the presence of CbrA-inducing signal(s), and so used a \(\text{crcZpa–gfp}\) fusion as a CCR bioindicator.

The expression of \(\text{crcZ}\) in the WT \(P. \text{aeruginosa}\) PAO1 strain was monitored in rich LB medium and in minimal media amended with various carbon sources. Fifteen different carbon sources were tested using 24-well plates (Figs 1a and S1a). In agreement with previous studies, \(\text{crcZ}\) expression was higher in minimal media amended with glucose than in those with succinate (Fig. 1) (Sonnleitner et al., 2009). The lowest \(\text{crcZ}\) expression was observed in the rich LB medium, and, as expected, in minimal media containing the C4-dicarboxylates fumarate, malate and succinate (Fig. 1a) (Valentini & Lapouge, 2013). Interestingly, the highest \(\text{crcZ}\) expression was observed in the presence of OAA, another C4-dicarboxylate and TCA cycle intermediate (Fig. 1a).

It is noteworthy that high expression of \(\text{crcZ}\) was observed when growth rate was low (i.e. OAA, arginine) or presented a long lag phase, thus showing accumulation of the gfp (i.e. histidine) (Figs S1a and S2a, Table 1). Inversely, in the presence of a repressor carbon source, where the growth rate was high (i.e. fumarate), \(\text{crcZ}\) expression showed lower levels (Fig. 1a, c). These results were in agreement with the general view that substrates yielding high energy and promoting rapid and fast growth were used preferentially, as a result of CCR.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>(P. \text{aeruginosa})</th>
<th>(P. \text{putida})</th>
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<tr>
<td></td>
<td>Generation time (h)</td>
<td>Lag phase (h)</td>
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<tr>
<td>LB</td>
<td>1.74</td>
<td>0</td>
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<tr>
<td>Fumarate</td>
<td>1.79</td>
<td>2</td>
</tr>
<tr>
<td>Malate</td>
<td>1.79</td>
<td>2</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.97</td>
<td>4</td>
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<tr>
<td>Succinate</td>
<td>2.02</td>
<td>2</td>
</tr>
<tr>
<td>Proline</td>
<td>2.13</td>
<td>4</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.23</td>
<td>4</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.3</td>
<td>4</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.45</td>
<td>2</td>
</tr>
<tr>
<td>Mannitol</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>4.05</td>
<td>4</td>
</tr>
<tr>
<td>Alanine</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Acetate</td>
<td>7.5</td>
<td>7</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>12.3</td>
<td>8</td>
</tr>
<tr>
<td>Arginine</td>
<td>&gt;13</td>
<td>&gt;13</td>
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</table>

To further demonstrate the hierarchy of carbon source utilization in \(P. \text{aeruginosa}\) PAO1, diauxic growth curves of the strain grown in minimal media amended with two different carbon sources were recorded. For that purpose, fumarate at low concentration (5 mM) was used as a repressing carbon source, in the presence of a non-preferential carbon source (i.e. glucose, citrate or histidine), to follow growth rate and de-repression of \(\text{crcZ}\) expression. The data showed hierarchical assimilation of the preferential (repressing) source initially and further assimilation of the non-preferential source upon exhaustion (Fig. S3a). Also, a clear induction of \(\text{crcZ}\) expression was detected at the same time as the fumarate was being depleted and the other carbon source (glucose or citrate) utilized (Fig. S3b). When using a combination of 5 mM histidine and 40 mM OAA, complete diauxic growth was not still visible after 800 min (Fig. S3b). Nevertheless, the growth rate observed indicated the assimilation of histidine at first, when compared with the growth curves in a minimal medium containing only one or the other carbon source. In agreement with a previous report (Sonnleitner et al., 2009), our results confirmed overall the direct correlation between the hierarchy of utilization of carbon sources with the activity of CbrA/B, i.e. \(\text{crcZ}\) expression.

**CbrA/B TCSs of \(P. \text{aeruginosa}\) and \(P. \text{putida}\) are fully exchangeable**

To investigate similarities and differences between the CbrA/B/CrcZ(Y) pathway in \(P. \text{aeruginosa}\) and \(P. \text{putida}\), we decided to analyse whether the TCS was able to induce similarly the expression of heterologous promoters. For this reason, we measured the activity of the \(P. \text{putida} \text{crcZ}\)

Table 1. Generation time and duration of latency (lag) phase of WT \(P. \text{aeruginosa}\) PAO1 and \(P. \text{putida}\) KT2442 growing on different carbon sources

Generation time was calculated during the exponential phase of growth as an extrapolation onto an exponential equation.
and crcY promoters (crcZpp and crcYpp, respectively) in P. aeruginosa and, conversely, the activity of the P. aeruginosa crcZ promoter (crcZpa) in P. putida. Bacteria were grown in minimal media supplemented with either OAA or fumarate as the sole carbon source, i.e. conditions where crcZpa expression showed the largest difference (Fig. 1). The highest expression of the chromosomal crcZpa–lacZ (PAO06816) reporter fusion and the plasmidic crcZpp–lacZ (pMPO1316) and crcYpp–lacZ (pMPO1314) reporter fusions was confirmed to be in the presence of OAA as compared with fumarate (Fig. 2). Furthermore, the activity level of all reporter fusions was equally comparable in both P. aeruginosa and P. putida strains. P. aeruginosa was able to activate the expression of crcY efficiently, even though there is no orthologue in this strain. These results showed that the CbrA/B TCSs of P. aeruginosa and P. putida can interchange target promoters and induce the expression of the downstream sRNA(s) gene(s) likewise.

Furthermore, the similarity of function and activity between the CbrA/B/CrcZ(Y) system of P. aeruginosa PAO1 and P. putida KT2442 is supported by their high sequence homology, reaching 82.19% amino acid identity for CbrA and 83.75% amino acid identity for CbrB. To further demonstrate that the CbrA sensor has the same function in both species, we swapped cbrA in the two species. We constructed PAO1ΔcbrA and KT2442ΔcbrA deletion mutants (PAO6862 and MPO413), and we tested their growth in BSM minimum medium supplemented with citrate, i.e. a carbon source that a cbrAB mutant is unable to utilize (Amador et al., 2010; Nishijyo et al., 2001). As expected, growth of both cbrA mutants was impaired in citrate, whilst Tn7 chromosomal insertion of cbrA from P. aeruginosa or P. putida, under the control of its own promoter (carried by pME10105 or pMPO1317, respectively), fully restored the growth of both mutants to the WT level (Fig. 3a, b). This result indicated that the CbrA proteins from P. aeruginosa and P. putida are fully interchangeable.

To substantiate our previous results showing that the expression of crcZpa, crcZpp and crcYpp was induced similarly in both Pseudomonas strains, we monitored the expression of the crcZpa–lacZ reporter fusion in P. aeruginosa and P. putida WT and cbrA mutant strains growing on fumarate, where all strains can grow (Fig. 3c). The expression of crcZ was abolished in both cbrA mutant strains and was restored to WT levels in cbrA mutants complemented with either the P. aeruginosa or P. putida cbrA gene.

**Carbon sources modulate crcZ expression similarly in P. aeruginosa and P. putida**

Whilst our previous results showed that both TCSs work similarly and that the regulatory elements are fully exchangeable between the species, in the literature, crcZ expression appeared to be different in P. aeruginosa versus P. putida depending on the carbon source used, e.g. succinate or glucose (Hester et al., 2000a; Moreno et al., 2012; Sonnleitner et al., 2009). Therefore, to investigate any possible difference between the signals regulating the CbrA/B/CrcZ(Y) systems of P. aeruginosa and P. putida, we monitored crcZ expression in a P. putida KT2442 WT strain growing in rich LB medium and in minimal media amended with various carbon sources as done above for P. aeruginosa PAO1 (Figs 1b, S1b and S2b). The same chromosomal crcZpa–gfp fusion, carrying the crcZpa promoter fused to the gfp, was also used as a reporter fusion in P. putida, as it was induced in a similar manner in both species (Fig. 2) and the promoters shared 61.2% sequence identity (Fig. S4). Interestingly, similar levels of crcZ expression were observed, i.e. high in OAA, low in fumarate/succinate and even lower in LB, and slight differences in the overall hierarchy of the carbon source utilization between the two species could also be observed, such as in histidine and glucose, due to accumulation during the lag phase (Fig. 1a, b). Similarly to P. aeruginosa, high crcZ expression correlated with a slow growth rate and/or presence of a long growth lag phase (Table 1). These results indicated once again that the CbrA/B system of P. aeruginosa and P. putida seemed to respond to the same signal(s) for the modulation of crcZ expression in response to different carbon sources. In both species, the CbrA/B cascade was highly active in OAA, whilst it was less active in fumarate/succinate and even less in LB. Taken together, all these results clearly indicated that the CbrA/B systems of
Investigation of the nature of the CbrA/B regulatory signal(s)

CCR in *Pseudomonas* was suggested to respond to the internal energy status of the cell rather than to external signals from the environment, as it is linked directly to the carbon metabolism of the bacterium (Duetz *et al.*, 1994, 1996; Rojo, 2010). However, the signal(s) activating the CbrA/B/CrcZ(Y) signal transduction pathway have not yet been deciphered. Therefore, we decided to search for internal molecules acting as a signal(s) for CbrA/B activity by carrying out a metabolomics approach.

We investigated the metabolome profiles of the WT strain PAO1 growing in flasks with minimal medium containing different carbon sources: OAA or fumarate/OAA. In the presence of fumarate, i.e. a preferentially metabolized carbon source by *P. aeruginosa*, CCR is active and therefore *crcZ* is expressed at a very low level. However, OAA, i.e. a non-energetically favourable carbon source (growth rate 3.5 h$^{-1}$ compared with 1.5 h$^{-1}$ in fumarate; Fig. 4), yields the highest induction rates of *crcZ* of all the media tested (Fig. 1). Diauxic growth was observed in the fumarate/OAA condition, indicating that CCR was occurring (Fig. 5). In this condition, two samples were collected: one after 2.5 h where fumarate was being used as the carbon source (CCR condition: point A; Fig. 4) and a second after 10 h where OAA was used (non-CCR condition: point B;...
Fig. 4). As a control condition, a third sample was taken from a PAO1 culture growing in minimal medium containing only OAA (non-CCR condition: point C, Fig. 4). To validate the CCR conditions used for the metabolome analysis, we measured the expression of the *crcZ*pa–*gfp* fusion in PAO1. As expected, *crcZ* expression was low in fumarate and was high as soon as the bacterium was using OAA (Fig. 5). In agreement, quantitative real-time PCR showed that the relative amount of *crcZ* transcripts in OAA was 4.1-fold higher than that in fumarate/OAA (data not shown).

In general, both conditions of carbon starvation (B and C) showed increased pools of amino acids compared with the carbon-excess condition (A; Fig. S5a). c-Aminobutyric acid was the only metabolite showing higher levels in condition A. Also, the detection of high levels of 2-OG in conditions B and C is striking (Fig. S7). As it is well known that the nitrogen status is signalled by the intracellular pools of glutamine (indicative of nitrogen sufficiency) and 2-OG (indicative of nitrogen limitation), and that the carbon/nitrogen ratio balance has been suggested as a signal for CbrA/B activation, we first evaluated the pools of such metabolites in conditions A, B and C. The amounts of both 2-OG and glutamine in condition A were below the limits of detection (Fig. S6, Table S3). The 2-OG/glutamine ratio was 0.5–0.67 for condition B, but reached 0.14 for condition C, indicating higher carbon starvation in the latter condition (Table S3).

The most noticeable difference in the amount of all the metabolites analysed when comparing conditions A and B or C was the abundance of glutamate, which was two orders of magnitude higher under carbon starvation (non-CCR) conditions. High levels of glutamate, together with the carbon limitation condition (or nitrogen excess) revealed by the 2-OG/glutamine ratio, could be due to a high activity of glutamate dehydrogenase, which is de-repressed by NtrC under these conditions (Herva&s et al., 2010).

**DISCUSSION**

In this work, we aimed at resolving the discrepancies observed between the CbrA/B/CrcZ(Y) signal transduction pathway in the two species *P. aeruginosa* and *P. putida*, and defining the signals activating this cascade.

To do so, we used the CbrA/B/CrcZ(Y) system as a readout for monitoring the carbon-source-specific response of pseudomonads. We first investigated the activity of the *P. aeruginosa* CbrA/B TCS on different carbon sources, using a *crcZ* reporter fusion as CCR indicator. *crcZ* expression (i.e. different CbrA/B activity) was observed to be modulated depending on the carbon source used (Fig. 1a). Among all the carbon sources tested, the highest induction of *crcZ* expression was in OAA or pyruvate, an intermediate level was detected in proline or lactate, and lower levels were seen in the TCA intermediates fumarate, malate and succinate. Finally, the lowest expression was found in the rich LB medium. Furthermore, a correlation between growth rate/lag phase length and induction of *crcZ* could be observed, as it is linked to CCR conditions, and the more energetically favourable a carbon source is (high growth rate and short lag phase), the more preferential it becomes.

Then, to decipher the differences between the CbrA/B/CrcZ(Y) pathway in *P. aeruginosa* and *P. putida*, we
investigated the ability of the TCS to induce the expression of the heterologous promoters. In the two Pseudomonas strains, CbrA/B not only activated similarly the expression of their own crcZ depending on the presence of different carbon sources, but also activated equally the transcription of the crcZ (and crcY) gene from the other strain (Fig. 2). Furthermore, we showed that the CbrA sensor is completely interchangeable between the two species when we functionally replaced the cbrA of P. aeruginosa PAO1 with the cbrA of P. putida KT2442 and vice versa (Fig. 3a, b). In addition, we showed that the expression of the crcZ promoter responds similarly to both P. aeruginosa and P. putida CbrA (Fig. 3c) even if slight differences in the overall hierarchy of the utilization of carbon sources could be observed. These variations in crcZ expression in the different media could reflect some differences in the carbon source uptake efficiency between the two species (Hoshino, 1998). Moreover, we also observed that crcZ expression in P. putida is modulated depending on the carbon source used, similarly as in P. aeruginosa, emphasizing the fact that the TCSs of both Pseudomonas species work similarly and that both TCSs respond to the same signals (Fig. 1).

In the literature, succinate has been used as a carbon source to observe CCR in P. aeruginosa and as the non-CCR condition in P. putida (Moreno et al., 2012; Sonnleitner et al., 2009). In this study, we used the same growth conditions for the two strains for the first time, in order to be able to compare them. By doing this, we showed that in both species, the crcZ (and crcY for P. putida) expression level is higher in succinate and citrate compared with LB. From our results, we can infer that the discrepancies for P. aeruginosa and P. putida in defining a carbon source as a CCR substrate depend on the utilization of different experimental conditions. Indeed, the carbon source hierarchy determined in this work showed that crcZ expression decreases from mannitol to citrate to succinate and then to LB in P. aeruginosa, and from citrate to succinate and then to LB in P. putida. Therefore, this hierarchy implies that succinate is a CCR source compared with mannitol and a non-CCR source compared with LB (Fig. 1).

Given the gradual response of the CbrA/B system to different carbon sources, it is unreasonable to propose that CbrA could sense every different carbon source and respond to them by modulating crcZ expression. For these reasons, we decided to investigate further what could influence CbrA activity (and therefore crcZ expression) in our conditions. The signal(s) regulating CbrA activity has been unknown, but was proposed to be related to the energy status of the cell. Therefore, to unravel the nature of the signal, we carried out a metabolomics approach. Metabolomic profiling represents a complementary approach to transcriptomics or proteomics to identify metabolic pathways and adaptation processes.

A direct observation from the metabolomic analysis is that the pool of amino acids is also increased under non-CCR conditions (B and C) compared with the fast-growing condition (A; Table S5). This reveals that, under these conditions, limitation of growth is not due to limited precursors for protein synthesis. An increased pool of amino acids could also illustrate the participation of the aminotransferases to transform o xo-acids into the corresponding amino acids (Fig. 6). γ-Aminobutyric acid, which is the only metabolite increased under conditions of CCR compared with the carbon-limited conditions, has been proposed as a physiological regulator of virulence and cytotoxicity in P. aeruginosa PAO1, Pseudomonas syringae and Pseudomonas fluorescens as it modulates biofilm formation activity and adhesion properties (Dagorn et al., 2013a, b; Park et al., 2010). Its production from glutamate through the glutamate decarboxylase system has also been implicated in acid tolerance in several bacterial genera facilitating intracellular pH homeostasis (Feehily & Karatzas, 2013). The physiological role of this observation should be further examined with other experimental approaches.

The energy content measured as the ATP/ADP ratio showed a higher ratio under carbon-limited conditions (1.21 ± 0.07 and 0.93 ± 0.03 for C and B, respectively) than condition A (0.43 ± 0.07), showing higher ATP availability under the conditions of higher carbon limitation (Fig. S8). This could reflect a slower ATP turnover and accumulation due to slow growth or the reduction of protein synthesis.

Accumulation of 2-OG when growing in OAA reveals deceleration or blockage of the TCA cycle in non-CCR conditions, probably at the level of 2-OG dehydrogenase activity (Fig. 6). The natural glutamate producer Corynebacterium glutamicum has taken advantage of this natural strategy through modification of the metabolic fluxes to yield higher levels of glutamate, by reduction of the 2-OG dehydrogenase complex activity that catalyses the conversion of 2-OG to succinyl-CoA (Fig. 6) (Asakura et al., 2007; Shirai et al., 2007).

[Image: Fig. 6. Diagram showing the TCA cycle, and the metabolites and enzymes involved in the 2-OG/glutamic acid/glutamine pathway in pseudomonads. Glu, glutamic acid; Gln, glutamine; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamine oxoglutarate amidotransferase.]
Glutamate is the metabolite with the most drastic changes in its abundance when the bacterium reaches carbon depletion (Fig. S6a). Glutamate is an important molecule for all living organisms and plays a pivotal role in various metabolic processes. It is involved in protein synthesis and other fundamental processes, such as glycolysis, gluconeogenesis and the TCA cycle. It is also a key metabolite as it serves to link nitrogen and carbon metabolism, and it is closely related to the TCA cycle and pathways assigned to core metabolism, thus positioning itself as a universal substrate for anabolic and catabolic processes. Glutamate in bacteria can be synthesized by two alternative routes. One route involves the reductive amination of 2-OG by glutamate dehydrogenase under conditions of nitrogen excess (>1 mM NH₄⁺), as it has a high Km for ammonium and this activity is repressed by NtrC (Hervás et al., 2010; Santero et al., 2012). The disadvantage of this pathway is its extra energy requirement. Nevertheless, when the ammonium concentration is lower, its assimilation can be also channelled by the participation of two enzymes: glutamine synthetase and glutamate synthase, also named glutamine oxoglutarate aminotransferase (Merrick & Edwards, 1995; Reitzer, 2003 and references within). When using OAA as carbon source, the glutamate dehydrogenase pathway is evidently favoured, as NtrC is inactivated and thus glutamate dehydrogenase activity totally unpressed, yielding high levels of glutamate from 2-OG (Fig. 6). An increased glutamate concentration in late growth phase has been reported previously in P. aeruginosa, its manifestation being independent of the growth conditions, composition of the media or genetic background (Frimmersdorf et al., 2010).

Overall, the results of our metabolomics experiment indicate that bacteria read carbon limitation as a low carbon/nitrogen ratio, which shows the close metabolic relationship between carbon and nitrogen availability. This ratio is known to control the activity of the NtrBC system through the uridylyl transferase PIII (Schumacher et al., 2013). Similar general indicators, such as the 2-OG/glutamine ratio, could also be used by the CbrA/B to respond to carbon availability.

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