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The global regulator Crc orchestrates the metabolic robustness underlying oxidative stress resistance in *Pseudomonas aeruginosa*

by

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SUMMARY. The remarkable metabolic versatility of bacteria of the genus *Pseudomonas* enable their survival across very diverse environmental conditions. *P. aeruginosa*, one of the most relevant opportunistic pathogens, is a prime example of this adaptability. The interplay between regulatory networks that mediate these metabolic and physiological features is just starting to be explored in detail. Carbon catabolite repression, governed by the Crc protein, controls the availability of several enzymes and transporters involved in the assimilation of secondary carbon sources. Yet, the regulation exerted by Crc on redox metabolism of *P. aeruginosa* (hence, on the overall physiology) had hitherto been unexplored. In this study, we address the intimate connection between carbon catabolite repression and metabolic robustness of *P. aeruginosa* PAO1. In particular, we explored the interplay between oxidative stress, metabolic rearrangements in central carbon metabolism and the cellular redox state. By adopting a combination of quantitative physiology experiments, multi-omic analyses, transcriptional patterns of key genes, measurement of metabolic activities *in vitro*, and direct quantification of redox balances both in the wild-type strain and in an isogenic Δ*crc* derivative, we demonstrate that Crc orchestrates the overall response of *P. aeruginosa* to oxidative stress via reshaping of the core metabolic architecture in this bacterium.

ORIGINALITY AND SIGNIFICANCE STATEMENT. Crc is considered to be a major player in the regulatory networks that rule the macroscopic physiology of many *Pseudomonas* species. Yet, a unifying explanation of the connection between carbon catabolite repression and the unique metabolic architecture of *P. aeruginosa* was largely missing, especially under environmental conditions compatible with the natural niches in which these bacteria thrive (usually characterized by the presence of different types of stressors). Our present study provides evidence that redox metabolism is the link between the regulation exerted by Crc and the metabolic robustness of *P. aeruginosa*. By using a combination of experimental approaches, we demonstrate how the multi-tiered regulatory pattern brought about by Crc mediates a re-shaping of the core metabolic network in *P. aeruginosa*—which in turn ensures a redox balance that empowers the cells to cope with oxidative stress. In all, our results indicate that while Crc is not indispensable for any essential cellular function, it decisively contributes to shape the environmental lifestyle of *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is an environmental bacterium characterized by a remarkable metabolic versatility that enables survival across a range of different (and often, extreme) conditions. These features are reflected in the capability of *P. aeruginosa* to colonize (and, sometimes, infect) different hosts—from plants to humans (Rahme *et al.*, 2000). As such, *P. aeruginosa* is one of the most relevant nosocomial opportunistic pathogens and is the main cause of chronic infections in cystic fibrosis patients (Folkesson *et al.*, 2012). The diverse niches in which *P. aeruginosa* thrives usually display steep gradients of nutrients (e.g. carbon and nitrogen sources) and O_2 (Palmer *et al.*, 2007; La Rosa *et al.*, 2018)—and bacterial cells often have to face other challenges simultaneously, such as the presence of multispecies microbiota and several types of stress factors, e.g. immune responses, antibiotics, and oxidative and osmotic stress (Moradali *et al.*, 2017). Sophisticated regulatory networks in Pseudomonads control the physiological responses to these stressful conditions, including both local and global regulators that adjust transcriptional levels according to environmental cues (Tribelli *et al.*, 2013; Balasubramanian *et al.*, 2015; Arce-Rodríguez *et al.*, 2016; Udaondo *et al.*, 2018). Carbon catabolite repression, governed by the Crc protein, is a major player among these regulatory systems in *Pseudomonas* species (Wolff *et al.*, 1991; Collier *et al.*, 1996; Velázquez *et al.*, 2004; Rojo, 2010; Grenga *et al.*, 2017; Liu *et al.*, 2017; Wirebrand *et al.*, 2018). Crc is a global regulator that exerts its influence at the post-transcriptional level by binding (together with the Hfq protein, the actual RNA-binding protein in the complex) a cognate region of target mRNAs (Kambara *et al.*, 2018), thus blocking translation (Moreno *et al.*, 2009; Milojevic *et al.*, 2013; Moreno *et al.*, 2015). The regulation pattern of Crc/Hfq also involves the action of the two-component system CbrAB and the small RNA CrcZ (**Fig. 1**). Recently published proteomic and transcriptomic studies have identified around 200 targets of Crc regulation in *P. aeruginosa* PAO1 (Reales-Calderón *et al.*, 2015b, a; Corona *et al.*, 2018). Although the effects of Crc seem to be pleiotropic, this regulator was found to control the availability of many enzymes and transporters involved in the assimilation of secondary C sources, similar to the situation described in *P. putida* (La Rosa *et al.*, 2015; La Rosa *et al.*, 2016). The impact of Crc on central carbon metabolism, on the other hand, is reflected in the regulation exerted by this protein on the levels of a few dehydrogenases and enzymes of the Entner-Doudoroff (ED) pathway (Wolff *et al.*, 1991; Reales-Calderón *et al.*,

2015b, a; Corona *et al.*, 2018). Yet, the overall physiological consequences of this regulatory pattern remain obscure.

Considering that environmental bacteria frequently face extreme conditions, which requires rapid and efficient responses to oxidative stress, it does not come as a surprise that their metabolic networks have evolved to rapidly react to such cues. This scenario is particularly relevant in the case of opportunistic pathogens as *P. aeruginosa*, since an efficient response to oxidative stress is a pre-requisite to counteract the activity of macrophages during infection. A rearrangement of the entire metabolic architecture of *Pseudomonas* species takes place to counteract an oxidative challenge, which could be summed up as the increase of metabolic activities that yield NADPH and the concomitant reduction of those that lead to NADH formation (Mailloux *et al.*, 2011; Nikel *et al.*, 2016; Lemire *et al.*, 2017). Several dehydrogenases of central carbon metabolism [e.g. glucose-6-*P* dehydrogenase (Zwf), isocitrate dehydrogenase (Icd), malic enzyme (Mae), and some glyceraldehyde-3-*P* dehydrogenases (Gap)] can generate NADPH during sugar catabolism (Spaans *et al.*, 2015). NADPH is a key player in the oxidative stress response (Ying, 2008), since it provides the reducing power needed to regenerate the active (reduced) form of many detoxifying enzymes. Glutathione reductase and thioredoxin reductase, for instance, both use NADPH to regenerate the active form of glutathione and thioredoxin that are used to quell reactive oxygen species (ROS) (Lu and Holmgren, 2014). Zwf is the main enzyme of central carbon metabolism concerning NADPH formation to counteract oxidative stress (Nikel and Chavarría, 2016). The production of this enzyme in *P. aeruginosa*, for instance, increases in response to oxidative stress, and its inactivation leads to susceptibility to such stress in most bacterial species (Ma *et al.*, 1998; Kim *et al.*, 2008; Sandoval *et al.*, 2011). Zwf [represented by three isozymes (Belda *et al.*, 2016)] is a key component of the EDEMP cycle of *P. putida* and *P. fluorescens*, which regenerates NADPH under glycolytic growth conditions (Nikel *et al.*, 2015a; Wilkes *et al.*, 2018), enabling a NADPH/$NADP^+$ balance needed to quench ROS by favoring the formation of the reduced nucleotide (Chavarría *et al.*, 2013). NADH formation, in contrast, is related to ROS generation because it serves as substrate of several membrane dehydrogenases (Esterhazy *et al.*, 2008). In this way, the NAPH/$NADP^+$ and NADH/NAD^+ redox balances are key factors of the overall response to oxidative stress in *Pseudomonas* (Mailloux *et al.*, 2011).

The tight regulation required to coordinate the assimilation of available C sources with a suitable energy balance that allows biomass formation while keeping the oxidative stress damage at bay in *P. aeruginosa* is a topic that keeps attracting attention as this is a critical aspect of niche adaptation. The interplay between catabolite repression control and the balance between ROS formation and oxidative stress responses, however, has not been explored in detail. The evidence gathered thus far suggest that the oxidative stress response could be altered in a *P. aeruginosa* Δ*crc* mutant (Linares *et al.*, 2010), yet the connection of this macroscopic phenotype with the regulation of central carbon metabolism and the cellular redox state remains largely uncharted. In this article, we have addressed the connection between the metabolic rearrangement brought about by ROS, the oxidative stress response, and Crc in *P. aeruginosa*. By adopting a combination of quantitative physiology experiments, multi-omic analyses, transcriptional examination of key genes responding to oxidative stress, measurement of metabolic activities *in vitro*, and direct quantification of redox balances both in the wild-type strain and in an isogenic Δ*crc* derivative, we demonstrate that Crc orchestrates the overall response of *P. aeruginosa* to ROS *via* reshaping of the core metabolic architecture of this bacterium.

RESULTS

Crc mediates tolerance of *P. aeruginosa* to oxidative insults. The starting point in assessing the role of Crc on the tolerance of *P. aeruginosa* to stressful conditions was evaluating the impact of exogenous oxidative stress on the cell physiology. To this end, the susceptibility to ROS of the wild-type strain PAO1 and the isogenic Δ*crc* mutant was firstly evaluated by means of a disk diffusion assay using different oxidative stress agents. The growth inhibition caused by treatment of the cells with either hydrogen peroxide (H_2O_2) or paraquat (PQ, *N*,*N*'-dimethyl-4,4'-bipyridinium dichloride, an oxidative agent that generates superoxide, $O_2^{\cdot-}$) were measured and compared (**Fig. 2A**). The Δ*crc* mutant was significantly more susceptible to oxidative stress caused by either oxidative stress agent than the parental strain. In particular, the inhibition zone observed after treatment of the cells with 1 µmol of H_2O_2 or 1 µmol of PQ increased by 1.3- and 1.5-fold, respectively, when comparing the Δ*crc* mutant with the wild-type strain.

Considering the increased susceptibility of the Δ*crc* mutant to oxidative stress, the involvement of the first line of cellular defense against oxidative stress (ROS detoxifying enzymes, e.g. catalases, superoxide dismutases, and alkyl hydroperoxide reductases) on the observed phenotypes was also investigated. The genome of *P. aeruginosa* PAO1 contains genes encoding catalases (*katA*, *katB*, *katE*, and *katN*), superoxide dismutases (*sodB* and *sodM*), and alkyl hydroperoxide reductases (*ahpC*, *ahpF*, *ahpB*, *ahpD*, and *PA_3529*). Among these detoxifying enzymes, those that have a proven functionality in *P. aeruginosa* are KatA, KatB, SodB, SodM, and AhpC. The total *in vitro* catalase (Kat) activity was evaluated in cell-free extracts of the wild-type strain and the Δ*crc* mutant grown in LB medium (**Fig. 2B**). Consistent with the high susceptibility to oxidative stress observed in the Δ*crc* mutant, this strain had a 1.8-fold increase in the total Kat activity when compared to the parental strain. This result indicates that, in the absence of the Crc regulator, *P. aeruginosa* had a high basal level of susceptibility to oxidative stress—which is reflected in a significant increase in the Kat activity even in the absence of any stressor. Prompted by this experimental observation, the oxidative stress defense of these strains was explored as indicated in the next section.

Transcriptional regulation of genes involved in the stress response. The expression of the main oxidative stress response genes of *P. aeruginosa* (*katA*, *katB*, *sodB*, *sodM*, and *ahpC*) was measured by real-time quantitative PCR (RT-qPCR) in order to test whether or not their expression was altered in the Δ*crc* mutant in comparison with the wild-type strain (**Fig. 3**). KatA is the constitutive *P. aeruginosa* catalase, accounting for most of the Kat activity during oxic growth of *P. aeruginosa*, whereas KatB is the main inducible catalase, counteracting exogenous oxidative stress (Brown *et al.*, 1995). In a similar fashion, SodB is the main superoxide dismutase and uses iron as a cofactor, while SodM is an inducible superoxide dismutase (Hassett *et al.*, 1997). AhpC is an alkylhydroperoxide, the transcript of which is detected in high quantities during an oxidative stress challenge (Hare *et al.*, 2011). On this background, the levels of gene expression were assessed in samples harvested during mid-exponential growth in LB medium, and compared to that of *rpoN* (*PA_4462*), considered as a housekeeping gene in these experiments. As indicated in **Fig. 3A**, the expression of *katA, sodM, sodB*, and *ahpC* did not present significant changes in the Δ*crc* mutant when compared to the wild-type strain. However, the expression of *katB* has a 3-fold increase in the Δ*crc* mutant. The expression of *katB* has been

previously shown to increase when strain PAO1 is exposed to an exogenous oxidative stress challenge (Ochsner *et al.*, 2000). Since *katB* was found to be overexpressed in the Δ*crc* strain during aerobic growth, this mutant seems to experience a higher basal level of endogenous oxidative stress than the parental strain—thus mirroring the results of the *in vitro* assessment of total Kat activity. Furthermore, examination of proteomic data of some of the key targets of OxyR-mediated regulation in *P. aeruginosa* PAO1 (Wei *et al.*, 2012) support the notion that the Δ*crc* strain undergoes a basal level of oxidative stress. KatB, AhpB, and AhpC, for instance, had a slight (but significant) increase in protein abundance levels [normalized $\log_2$(Δ*crc*/PAO1) > 0.3].

The level of expression of key genes encoding ROS defense functions was also measured in response to an oxidative stress challenge with H_2O_2. To this end, the wild-type strain and its Δ*crc* derivative were grown in LB medium as indicated above, and H_2O_2 was added at 1 mM in some of these cultures. The expression of *katA, katB, sodM*, and *ahpC* was significantly induced in the presence of H_2O_2 in the wild-type strain (**Fig. 3B**), with increases in the level of gene expression that ranged from 52-fold (*ahpC*) up to 491-fold (*sodM*) as compared to the control condition (i.e. LB medium). The expression of the same genes was likewise induced in the Δ*crc* strain by treatment with H_2O_2; however, the induction levels for the whole set of genes were slightly lower than those in the wild-type strain. For example, the oxidative stress challenge induced *katA* expression by 41-fold in the Δ*crc* strain and by 293-fold in the wild-type strain. The expression level of *sodB*, in contrast, was not altered by the oxidative stress challenge—and it was actually slightly repressed both in strain PAO1 and in the Δ*crc* mutant. In all, the exploration of the transcriptional landscape of these strains by RT-qPCR revealed that the Δ*crc* mutant undergoes a condition of constitutive oxidative stress not observed in the wild-type strain. Yet, what is the origin of this endogenous stress in the Δ*crc* mutant?

Elimination of Crc in *P. aeruginosa* leads to high respiration rates and superoxide formation, low cell vitality and increased pyocyanin biosynthesis. Considering that ca. 90% of the ROS formed in bacteria under oxic growth conditions are generated by the electronic transport chain (ETC) (Scialò *et al.*, 2017; Maklashina *et al.*, 2018), the specific rates of respiration and $O_2^{\cdot -}$ formation were assessed in the strains under study (**Fig. 4**). When the specific rates of respiration were measured by using a Clark-type electrode in cells sampled

during mid-exponential growth from LB medium cultures, a 1.4-fold increase in the parameter was observed in the Δ*crc* mutant as compared to the wild-type strain (**Fig. 4A**). Such significant increase in O_2 consumption in the mutant strain could correlate to enhanced $O_2^{\cdot-}$ formation, and a biochemical test was adopted to quantify $O_2^{\cdot-}$ by means of the redox-sensitive compound nitro-blue tetrazolium chloride (NBT). To this end, NBT reduction was quantified in both *P. aeruginosa* PAO1 and its Δ*crc* derivative under the same growth conditions indicated above (**Fig. 4B**). The formation of $O_2^{\cdot-}$ in this experiment followed the same trend as the specific rates of O_2 consumption, i.e. the Δ*crc* mutant had a 1.5-fold increase in ROS accumulation as compared to *P. aeruginosa* PAO1. When the cells were exposed to an oxidative stress challenge by addition of PQ to the culture medium at 1 mM, a significant increase was observed in $O_2^{\cdot-}$ formation in the two strains—yet, the Δ*crc* mutant had a larger increase in ROS accumulation upon exposure to PQ. Interestingly, the Δ*crc* mutant growing in the absence of any stressor showed levels of $O_2^{\cdot-}$ formation comparable to *P. aeruginosa* PAO1 exposed to PQ.

The results of respirometry and of the biochemical analysis of ROS formation support the notion that the Δ*crc* mutant is exposed to a basal condition of oxidative stress. If this is the case, the general cell vitality and metabolic activity, i.e. catalytic vigor (Nikel *et al.*, 2013), should be likewise affected in the mutant. To test this hypothesis, the chemical sensor *Bac*Light™ RedoxSensor™ Green (RSG) was employed to measure the total reductase activity of bacteria (**Fig. 4C**). The green fluorescence arising when RSG is oxidized serves as a gauge of the total dehydrogenase activity in the cells, and therefore the cell vitality (Kalyuzhnaya *et al.*, 2008). In line with the results described above, the Δ*crc* mutant had a lower cell vitality in comparison with the wild-type when growing on LB medium (88% with respect to the wild-type strain). As an additional control, cells were separately treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which is an ionophore that dissipates the proton gradient of the plasmatic membrane (ΔpH), thus lowering the overall metabolic activity. The CCCP treatment caused a reduction in the total metabolic activity of the cells in both bacterial strains—in *P. aeruginosa* PAO1, a 45% decrease in the RSG fluorescence was observed upon addition of the ionophore, but the reduction in metabolic activity was even higher in the Δ*crc* mutant (i.e. a 80% decrease as compared to the untreated control).

Apart from the activity of the respiratory chain as a source of $O_2^{\cdot-}$ and NADH oxidation, redox-active secondary metabolites, known as phenazines, act as electron carriers and have been demonstrated to affect the redox balance in *P. aeruginosa* (Turner and Messenger, 1986; Lau *et al.*, 2004). Pyocyanin (5-methylphenazin-1-one), for instance, has a prominent role in modulating the intracellular redox state of *P. aeruginosa* PA14 by lowering the intracellular NADH/NAD^+ ratio (Price-Whelan *et al.*, 2007). We measured the amount of pyocyanin excreted by the strains under study in LB medium cultures, and found that the Δ*crc* mutant had a 1.4-fold increase in pyocyanin biosynthesis as compared with the wild-type strain after 24 h of incubation. Consistent with the increase in respiratory chain activity observed in the mutant, this phenomenon suggests an alternative mechanism for balancing the redox status of the cells. Taken together, the results above indicate that the absence of Crc leads to higher respiration rates, which correlate with increased generation of ROS, pyocyanin secretion and a concomitant decrease in the overall metabolic robustness of *P. aeruginosa*. On this background, the metabolic mechanisms at play under these conditions were investigated as explained below.

Quantitative transcriptomic and proteomic analyses expose the role of Crc in re-shaping central carbon metabolism of *P. aeruginosa*. Previous studies highlighted several targets for Crc in the genome of *P. aeruginosa* PAO1, with a predominant role of the regulator in controlling the availability of enzymes and transporters involved in the assimilation of secondary C sources. In addition, the results of the previous sections indicate that a Δ*crc* mutant is subjected to a basal level of elevated stress due to increased respiration and $O_2^{\cdot-}$ formation. In order to shed light on the relationship between Crc and redox metabolism, we inspected the biochemical network of *P. aeruginosa* and mapped the nodes that showed a differential pattern of abundance both at the level of mRNA and protein when comparing the Δ*crc* mutant against the wild-type strain. We gathered data both from RNA sequencing and isobaric tags for relative and absolute quantitation (*i*TRAQ)-based proteomics (Reales-Calderón *et al.*, 2015b; Corona *et al.*, 2018) in exponentially-growing LB medium cultures, in which the Crc-dependent catabolite repression is maximal (Valentini *et al.*, 2014), for *P. aeruginosa* PA01 and its Δ*crc* derivative (**Fig. 5**). The genes that had a significantly different level of transcription between the two strains were found to be repressed in *P. aeruginosa* Δ*crc*. In particular, *zwf* (*PA_3183*), *edd* (*PA_3194*), *eda* (*PA_3181*), *PA_2265* (encoding Gcd, a gluconate dehydrogenase), and *PA_1252* (encoding malate

dehydrogenase) had lower levels of transcription in the mutant strain than in the parental *P. aeruginosa* PAO1 strain—although the transcriptional repression was relatively mild, with –2 < normalized $\log_2$(Δ*crc*/PAO1) < –1 for all the genes detected.

The control exerted by Crc on central carbon metabolism was much more evident at the protein level (**Fig. 5**). When the abundance of key proteins in the biochemical network was analyzed, an increase was detected in the Δ*crc* strain in Zwf, Edd, Eda, and, to a lesser extent, in Glk (PA_3193), GapA (PA_3195), PycB (PA_5435), and FumC1 (PA_4470). Interestingly, the pattern of transcriptional and post-transcriptional regulation in some of these metabolic nodes (e.g. in the glucose-6-*P* dehydrogenase step) followed opposite trends, which highlights the importance of combining the analysis of the two regulation levels (i.e. mRNA and protein abundance) when studying the pleiotropic effects brought about by the Crc regulator. Finally, Zwf, Edd, Eda, GapA, Pgl, Glk, Gcd, PycAB, AceE and FumC1 are described to be post-transcriptional targets of Crc regulation. In all, this pattern of regulation suggests increased catabolism in *P. aeruginosa* in the absence of Crc through the sequence of reactions Glk (sugar phoshorylation) → Zwf [first step of the pentose phosphate (PP) pathway] → Edd/Eda (catabolic Entner-Doudoroff pathway). This pattern of catabolic activities suggests a role of the upper domain of the biochemical network in the Δ*crc* mutant that goes beyond sugar utilization (the availability of which would be relatively low in complex LB medium). A deep biochemical characterization of these metabolic nodes in *P. aeruginosa*, as explained in the next section, provided a connection between metabolic activity and redox metabolism.

The absence of Crc mediates increased activities through the upper catabolic domain in *P. aeruginosa*. The evidence gathered so far indicates that there is a high basal level of oxidative stress in a Δ*crc* mutant of *P. aeruginosa*, which translates into the constitutive activation of detoxifying enzymes even in the absence of external stressors. Concomitant with these phenomena, a re-arrangement of the biochemical network would suggest that Crc controls key nodes in catabolism. In order to shed light on this aspect, we measured the enzymatic activities of the main enzymes affected at the proteomic level by means of specific *in vitro* assays in cell-free extracts of the strains grown in LB medium (**Fig. 6**). The activity of the ED pathway was assessed by a combined assay for Edd (6-phosphogluconate dehydratase) and Eda (2-dehydro-3-

deoxygluconate-6-*P* aldolase), in which pyruvate (one of the final products of ED pathway) is quantified by coupling the assay to the activity of lactate dehydrogenase. The two activities of the ED pathway had a significant increase (5-fold change) in the Δ*crc* strain as compared with the wild-type strain (**Fig. 6A**). When the total $NADP^+$-dependent glucose-6-P dehydrogenase activity was assayed in vivo, a similarly high increase (7-fold) was observed in the mutant strain (**Fig. 6B**).

The changes in the levels of Edd/Eda and Zwf activity observed in the *in vitro* assays are consistent with the increase in the abundance of the corresponding proteins detected in the Δ*crc* mutant (**Fig. 5**). In particular, the significant increase in the Zwf activity prompted the question of whether the role of this enzyme in providing NADPH could be relevant for maintaining the redox balance in the mutant strain. Moreover, since the catalytic function of ROS detoxifying enzymes relies on the reducing power, and NADPH is needed to regenerate the active (reduced) form of the enzymes in each catalytic cycle, Zwf could help providing such reducing power. This possibility was tested by exposing both the wild-type strain and the Δ*crc* mutant to oxidative stress conditions externally imposed by addition of PQ (**Fig. 6C**). The *in vitro* Zwf activity rose by 1.5-fold in the parental strain in response to the oxidative stress challenge. In the case of the Δ*crc* mutant, in contrast, the Zwf activity did not suffer any major changes in the oxidative stress situation, and remained within the (very high) levels observed in control cultures without PQ. Thus, it seems that the Δ*crc* mutant is unable to modulate the production of Zwf in response to an oxidative stress challenge over its abnormally high values. In a broader context, the increase in the fluxes through the ED pathway and the first step of the PP pathway should affect the redox balance in the mutant strain—a scenario that was explored by direct measurement of pyridine nucleotides as indicated below.

The metabolic imbalance brought about by Crc elimination affects the redox status of *P. aeruginosa* at the level of NADPH availability. The cellular redox status was assessed in the strains under study by directly quantifying the redox ratios at the level of the pyridine nucleotide cofactors NAD^+, NADH, $NADP^+$, and NADPH using a cycling biochemical assay *in vitro*. Under these experimental conditions, i.e. mid-exponential growth on LB medium under oxic conditions, the NADH/NAD^+ ratio had comparable values (ca. 0.8 mol · mol^{-1}) in both the parental and the

Δ*crc* mutant strains (**Fig. 7**). The NADPH/NADP$^+$ ratio, in contrast, was significantly higher (1.8-fold) in the Δ*crc* mutant than in *P. aeruginosa* PAO1, and this increase in the anabolic charge of pyridine nucleotides was also reflected in the overall NAD(P)H/NAD(P)$^+$ ratio (which includes all the redox cofactors). These results are expose a higher content of NADPH in the Δ*crc* strain—arising, in turn, from substantially increased fluxes through Edd/Eda and (especially) the NADP$^+$-dependent Zwf dehydrogenase, and correlating with an increased demand of this reduced nucleotide needed to counteract oxidative stress.

DISCUSSION

Over the years, several studies exploring the role of Crc clearly indicated that this global regulator influences several cellular functions at the post-transcriptional level in Pseudomonads (La Rosa *et al.*, 2016), but a unifying theory about how Crc coordinates the redox response and central carbon catabolism is still largely missing. In this work, we have demonstrated that the regulation exerted by Crc in *P. aeruginosa* bridges the redox metabolism and the oxidative stress response by modulating the metabolic fluxes through NADP$^+$-dependent dehydrogenases. NADPH, formed in the upper domain of the biochemical network, helps counteracting a basal level of oxidative stress experienced by the Δ*crc* mutant strain.

Many *Pseudomonas* species are aerobes, and one of the main consequences of the bacterial oxic metabolism is the generation of ROS, e.g. $O_2^{\cdot -}$, which can cause damage to the cell by indiscriminately oxidizing macromolecules such as DNA or proteins (Imlay, 2013). *Pseudomonas* species employ several (and often, concomitant) mechanisms to detoxify ROS (Kim and Park, 2014): (i) increasing the formation of specific ROS detoxifying enzymes (Lu and Holmgren, 2014), (ii) controlling other physiological aspects related to ROS production, such as iron homeostasis (Lemire *et al.*, 2017), and (iii) rewiring central carbon metabolism to favor reducing power homeostasis (Nikel and Chavarría, 2016). Our results indicate that Crc modulates all three aspects in *P. aeruginosa* at different levels.

The oxidative stress response has been shown to be deregulated in a Δ*crc* mutant, and this strain is considerably more susceptible to oxidative stress agents than its parental counterpart

(**Fig. 2**). Secondly, in such conditions, the Δ*crc* strain had higher levels of $O_2^{\cdot-}$ formation, linked to an increase in the specific O_2 uptake rate (**Fig. 4**). Data from the literature indicates that this mutant also exhibits higher levels of intracellular iron, which catalyzes the ROS-generating Fenton reaction (Corona *et al.*, 2018), and the production of a great number of membrane vesicles that could be caused by oxidative stress is likewise increased in the absence of Crc (Sabra *et al.*, 2003; Reales-Calderón *et al.*, 2015b). It is also likely that this phenotype involves a certain degree of uncoupling of oxidative phosphorylation, which occurs when the activity of the ETC and the synthesis of ATP do not occur in a balanced manner, e.g. due to an increase of the activity of the less efficient terminal oxidases of the ETC (Kadenbach, 2003). This unbalanced activity of the ETC components leads to elevated ROS formation and high rates of O_2 consumption (Lamprecht *et al.*, 2016). Supporting this notion, significant changes have been detected in the composition of some of the five ETC terminal oxidases in the Δ*crc* mutant (Reales-Calderón *et al.*, 2015b; Corona *et al.*, 2018), which correlates with the results of the RSG staining assay (**Fig. 4**).

One way or the other, the relevant question related to the observed phenotypes is how do the cells cope with this endogenous stress situation in terms of metabolic rewiring? Metabolic fluxes play an essential role in the maintenance of adequate levels of NADPH to cope with oxidative stress, and several enzymes of the central carbon metabolism generate NADPH, e.g. Zwf, Icd (isocitrate dehydrogenase), and Gnd (6-phoshogluconate dehydrogenase) (O'Toole *et al.*, 2000; Nikel *et al.*, 2015a; Spaans *et al.*, 2015). In the present study, we have detected a significant increase in the fluxes through the ED pathway and Zwf in the absence of Crc, both at the level of protein abundance (**Fig. 5**) and *in vitro* measurement of enzymatic activities (**Fig. 6**). Other dehydrogenases have been shown to be relevant for maintaining the redox balance in *Pseudomonas* species upon exogenous oxidative challenges, e.g. isocitrate dehydrogenase and 6-phosphogluconate dehydrogenase. Two genes encoding potential isocitrate dehydrogenase activities are present in *P. aeruginosa*, i.e. *icd* (*PA_2629*) and *idh* (*PA_2624*). This enzyme belongs to the tricarboxylic acid cycle and catalyzes the $NAD(P)^+$-dependent conversion of isocitrate into α-ketoglutarate. The total Icd activity was assayed in the strains under study, and a small decrease could be detected in the Δ*crc* strain (data not shown), which is consistent with the general downregulation of biochemical activities that generate NADH and the concomitant

increase in fluxes that lead to NADPH formation when *Pseudomonas* species are exposed to exogenous oxidants. Interestingly, we could not detect any 6-phosphogluconate dehydrogenase (Gnd, an enzyme of the oxidative PP pathway) activity in the cell-free extracts of the wild-type strain or its Δ*crc* derivative. Although this enzyme has been included in some metabolic models of *P. aeruginosa*, we could not detect any Gnd activity in the strains, and no orthologue of *gnd* of *P. putida* KT2440 could be found in the PAO1 genome. Moreover, the metabolic rewiring necessary to cope with the elevated levels of endogenous oxidative stress in the Δ*crc* strain rendered the NADPH-generating Zwf activity unresponsive to further (external) stress (**Fig. 6**). NADPH regeneration through alternative biochemical mechanisms independent of catabolism, such as transhydrogenation (Fuhrer and Sauer, 2009), are equally possible. We have detected a slight increase in the protein abundance of PntA (PP_0195), a subunit of the membrane bound transhydrogenase of strain PAO1 and part of the OxyR regulon, which catalyzes the NADH → NADPH transhydrogenation reaction. Transhydrogenation has been shown to be a relevant mechanism for NADPH regeneration in *Pseudomonas* species subjected to oxidative stress (Decorosi *et al.*, 2011; Nikel *et al.*, 2016). Furthermore, the increase observed in pyocyanin excretion could also contribute to favor a higher $NADPH/NADP^+$ ratio as compared to the $NADH/NAD^+$ ratio in the Δ*crc* strain, as previously suggested by Price-Whelan *et al.* (2007).

In summary, the physiological situation characteristic of the Δ*crc* mutant implies an increase in the basal level of oxidative stress with respect to the parental strain, an inefficient response to external oxidative stress challenges, and probably some degree of uncoupling in the oxidative phosphorylation. Taken together, these results highlight that the Crc regulator is necessary for the maintenance of the metabolic robustness of *P. aeruginosa*, allowing for an adequate response to environmental stresses, thus enabling adaptation to colonize different habitats, including the host during the infective process.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions. The bacterial strains used in this study were the wild-type strain *P. aeruginosa* PAO1 (Holloway, 1969), a generous gift of P. V. Phibbs, and its isogenic, marker-less Δ*crc* mutant previously described by Reales-Calderón *et al.* (2015b).

Bacterial strains were grown in complex LB medium (10 g · liter^{-1} tryptone, 5 g · liter^{-1} yeast extract, and 5 · g liter^{-1} NaCl; Pronadisa Ltd., Spain) at 37°C with constant shaking at 250 rpm. Inocula were prepared by growing the cells in the same conditions, and working cultures were developed in 20 ml of culture medium in 100-ml Erlenmeyer flasks inoculated at an initial optical density measured at 600 nm (OD_{600}) of 0.05. Quantitative physiology experiments and transcriptional analysis were carried out with cells harvested at the mid-exponential phase of growth (when OD_{600} = 0.5).

RT-qPCR–based transcriptional analysis of genes involved in the oxidative stress response. Oligonucleotides used in this work are listed in **Table 1**. Samples were obtained by centrifuging 20-ml aliquots of the bacterial culture (5,800×*g*, 20 min, 4°C). Pellets were immediately frozen by immersion of the tubes in liquid N_2, and kept at –80°C for up to 1 month until processing. By the time of processing, lysozyme was added to the thawed bacterial pellets at a final concentration of 1 mg · ml^{-1}. Then, the samples were incubated during 10 min at room temperature with intermittent vortexing and sonicated on an ice bath in 3 cycles of 20 s at a power output of 0.4 Hz followed by a rest step of 20 s. RNA extraction was carried out using the *RNA Easy Minikit*™ (Qiagen Inc., USA). DNA was removed using the *RNase-free DNAse* set provided in the kit, and the samples were further subjected to treatment with the *Turbo DNA-free* system (Ambion™ for Thermo Fisher Scientific Inc., USA) to remove any possible remaining traces of contaminating DNA. RNA samples were routinely checked for DNA contamination through PCR with oligonucleotides *rplU*_F and *rplU*_R (**Table 1**). cDNA was synthesized with the *High Capacity cDNA Reverse Transcriptase Kit* (Applied Biosystems™ for Thermo Fisher Scientific Inc., USA). RT-qPCR was performed with 50 ng of cDNA per reaction using the *Power-Green PCR Master Mix* in a 7500 Real Time PCR System thermocycler (Applied Biosystems™). Gene expression levels were quantified by using the $2^{-\Delta\Delta CT}$ method as previously described (Livak and Schmittgen, 2001). The expression levels of *rpoN* (*PA_4462*, considered to be a housekeeping gene in this study) were used to compare transcriptional values among different conditions and strains.

Omics data acquisition, processing and availability. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Deutsch *et al.*, 2017) *via* the PRIDE

partner repository (Vizcaíno *et al.*, 2016) with the dataset identifier PXD009809 (Reales-Calderón *et al.*, 2015b, a). Raw data of RNA sequencing experiments is available in Corona *et al.* (2018). Data analysis and acquisition was performed as indicated by Kim *et al.* (2013) and Corona *et al.* (2018).

Oxidative stress susceptibility test. To quantify the susceptibility of the strains to oxidative stress, bacterial cultures, harvested during mid-exponential growth, were diluted to OD_{600} = 0.005 in 0.85% (wt/vol) NaCl. Hundred microliters of this dilution were seeded onto a LB agar plate and were let to dry. Immediately after, 1 μmol of either H_2O_2 or paraquat were added to 9-mm sterile filter paper disks (Macherey-Nagel GmbH & Co. KG, Germany), which were placed onto each plate, from freshly-prepared concentrated solutions. The plates were incubated at 37°C during 20 h. The diameter of the growth inhibition halos caused by the presence of oxidative stressors was measured using the Java-based ImageJ software (Schneider *et al.*, 2012) from pictures taken from each plate. The experiment was performed in independent triplicates.

Cell-free extract preparation and *in vitro* determination of enzymatic activities. Bacterial cell-free extracts were prepared as follows. First, 10-ml aliquots of each culture, harvested during mid-exponential growth, were centrifuged (5,800×*g*, 3 min, 4°C) and the bacterial pellets were washed twice with the same volume of cold 50 mM phosphate buffer (pH = 7.5). Cell-free extracts were obtained by sonication on an ice bath (3 cycles of 20 s at 0.4 Hz, with stop rests of 20 s in-between sonication steps). The concentration of total proteins in the cell-free extracts was quantified using the *Pierce BCA (bicinchoninic acid) Protein Assay Kit* (Thermo Fisher Scientific Inc.). All the enzymatic activities described below were normalized by the total amount of proteins in the cell-free extract and expressed as specific activities in U of activity · $mg_{protein}^{-1}$. All enzymatic determinations were carried out with cell-free extracts obtained from at least three independent cultures.

In vitro determinations of enzymatic activities followed previously developed protocols (Shin *et al.*, 2008; Nikel *et al.*, 2014; Dvořák *et al.*, 2015; Benedetti *et al.*, 2016; Nikel and Chavarría, 2016), with the modifications described in this section. Catalase (EC 1.11.1.6) was assayed by mixing 5 μl of the cell-free extracts with 1 ml of 10% (vol/vol) H_2O_2 as the reaction substrate in a quartz

cuvette. The absorbance at 240 nm (A_{240}) was recorded every second during 2 min upon addition of the cell-free extracts to the reaction mixture. A linear regression of A_{240} *versus* time was used to determine the rate of H_2O_2 decomposition, and a molar extinction coefficient $\varepsilon = 43.6\ M^{-1} \cdot cm^{-1}$ was employed to calculate the specific catalase activity.

Glucose-6-*P* 1-dehydrogenase (Zwf, 1.1.1.49), isocitrate dehydrogenase (Icd, EC 1.1.1.42), and 6-phosphogluconate dehydrogenase (Gnd, EC 1.1.1.44) were assayed by coupling the activities with the measurement of NADPH formation. Ten microliters of the corresponding cell-free extracts were mixed with 90 μl of the following reaction mixtures (all concentrations indicated are final): (i) Zwf activity; 50 mM phosphate buffer (pH = 7.5), 10 mM $MgSO_4$, 0.75 mM $NADP^+$, and 2 mM glucose-6-*P*; (ii) Icd activity; 67 mM phosphate buffer (pH = 7.5), 0.6 mM $MnCl_2$, 1 mM $NADP^+$, 0.44 mM D,L-isocitric acid; and (iii) Gnd activity; 94 mM phosphate buffer (pH = 7.5), 2 mM $NADP^+$, and 1.7 mM 6-phosphogluconate. In all cases, NADPH formation was measured by tracking the absorbance at 340 nm in multi-well microtiter plates using an *Infinite M200* microplate reader (Tecan Group Ltd., Switzerland). An extinction coefficient for NADPH $\varepsilon = 6.22\ mM^{-1} \cdot cm^{-1}$ was used to calculate the rate of NADPH formation. All the activities were calibrated with purified enzymes (Sigma-Aldrich Co., USA), and a cell-free extract of glucose-grown *P. putida* KT2440 was included as a positive control for the Gnd activity (Nikel *et al.*, 2015a).

Enzymatic activities of the ED pathway [6-phosphogluconate dehydratase (Edd, EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (Eda, EC 4.1.2.14)] activities were evaluated in a two-step reaction assay. The 100-μl assay comprised 90 μl of a reaction mixture (composed of 50 mM Tris·HCl (pH = 7.5), 10 mM $MgCl_2$, 100 μM 6-phosphogluconate) and 10 μl of the cell-free extract. After an incubation step of 30 min at room temperature, the mixture was diluted in the same reaction mixture to 1 ml, and was heated at 95°C during 2 min. The resulting solution was centrifuged (12,000×*g*, 10 min, room temperature) and pyruvate (end product of the combined action of Edd and Eda) was quantified in the supernatant through an LdhA-dependent approach (Nikel *et al.*, 2010). To this end, a 100-μl aliquot of the supernatant was mixed with a second reaction mixture containing 50 mM Tris·HCl (pH = 7.5), 10 mM $MgCl_2$, 0.1 mM NADH, and 0.5 U of L-lactate dehydrogenase (Roche™ for Sigma-Aldrich Co.) in a final volume of 1 ml. The resulting reaction was incubated at 37°C and the absorbance at 340 nm was recorded over

time similarly to the previously described assays. The resulting pyruvate concentrations (and formation rates) were calculated with an extinction coefficient for NADH ε = 6.22 $mM^{-1} \cdot cm^{-1}$.

***In vitro* quantification of pyridine nucleotides, redox ratios and pyocyanin concentration.** Pyridine nucleotides [NAD(P)/(+/H)] were determined by following the cyclic method described by Bernofsky and Swan (1973) as adapted to *Pseudomonas* (Price-Whelan *et al.*, 2007; Nikel and Chavarría, 2016), with minor modifications. Six 2-ml aliquots were promptly harvested from mid-exponential cultures and centrifuged (12,000×*g*, 1 min, –4°C), the supernatant was removed and the bacterial pellets were immediately frozen by immersion in liquid N_2. Three hundred microliters of 0.2 M NaOH [for NAD(P)H extraction] or 300 μl of 0.2 M HCl [for $NAD(P)^+$ extraction] were added to the frozen samples and the resulting mixtures were incubated during 10 min at 55°C, followed by a 10-min incubation on an ice bath. Three aliquots per conditions (either acidified or basified) were combined in a single 2-ml Eppendorf tube and cellular debris were removed by centrifugation (12,000×*g*, 5 min, 4°C). Five microliters of the resulting supernatants were mixed with 90 μl of a reaction mixture containing (final concentrations in a final volume of 100 μl): 120 mM bicine·NaOH (pH = 8.0), 0.5 mM MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], 4.5 mM EDTA (ethylenediaminetetraacetic acid), 4.5 mM phenazine ethosulfate, and the appropriate substrate for each type of chemical species: 200 mM ethanol for NAD(+/H) determinations and 12.5 mM glucose-6-*P* for NADP(+/H) determinations. Ninety-five microliters of the resulting mixture were placed in a microtiter plate, and the reaction was started with the addition of 5 μl of an alcohol dehydrogenase suspension at 350 $U \cdot ml^{-1}$ [for NAD(+/H) determinations] or glucose-6-*P* dehydrogenase (Sigma-Aldrich Co.) at 5 $U \cdot ml^{-1}$ [for NADP(+/H) determinations]. A calibration curve was stablished with known concentrations of each pyridine nucleotide, ranging from 0.015 to 1 mM. Reactions were performed at 30°C, and the absorbance at 570 nm (A_{570}) was recorded every 20 s during 10 min for each assay. The slope of ΔA_{570} versus time was obtained for individual reactions in order to calculate the concentration of each nucleotide in the samples. Blank values were subtracted, and the $\Delta A_{570}/\Delta t$ values of the calibration curve reactions were represented as a function of the nucleotide concentration. Using the resulting calibration curves, the absolute concentration of each nucleotide in the samples was calculated, and the corresponding redox ratios were obtained as [NADH]/[NAD^+], [NADPH]/[$NADP^+$], and ([NADH]+[NADPH])/([NAD^+]+[$NADP^+$]).

The formation of the blue pigment pyocyanin was determined as indicated in Sánchez *et al.* (2002) by measuring the absorbance of filtered culture supernatants at 690 nm. In these experiments, cells were grown in LB medium for 24 h as indicated above. At this time, 1 ml of each culture was centrifuged (12,000×*g*, 5 min, 4°C) and the supernatants were filtered through a 0.22-μm pore filter. Each experiment was performed in triplicate, and results were averaged after normalizing the absorbance readings to the cell density in each sample.

Superoxide anion formation. The protocol described by Pérez-Pantoja *et al*. (2013), which is based on the reduction of NBT, was adopted to quantify the formation of superoxide anion. For this, 1.5-ml aliquots of the corresponding culture harvested during the mid-exponential growth phase were incubated with NBT at 350 $\mu g \cdot ml^{-1}$ during 30 min at 37°C in the dark, and HCl was added at a final concentration of 7.5 mM to lyse the cells after this incubation step. Then, samples were centrifuged (2,500×*g*, 15 min, room temperature) and bacterial pellets were treated twice with 350 μl of dimethyl sulfoxide to extract the reduced NBT. Supernatants were recovered, pooled in a clean Eppendorf tube and mixed with 1 ml of 50 mM phosphate buffer (pH = 7.5). The absorbance at 525 nm (A_{525}), which correlates with the color change between the oxidized and reduced forms of NBT, was measured in all the samples. The A_{525} values were normalized with the cell density of each sample (OD_{600}) and were expressed as the fold-change with respect with the wild-type strain.

Determination of the oxygen consumption rate. The rate of oxygen consumption by the strains under study was measured using an *Oxygraph* oximeter (Hansatech Instruments Ltd., United Kingdom). A Clark-type electrode ($O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$) was used according to the manufacturer's instructions. Firstly, 900 μl of LB medium, pre-heated at 37°C, were promptly added to the measurement chamber after vigorous shaking to ensure the highest oxygen saturation in the liquid phase. Then, 100 μl of a mid-exponential culture were added to the chamber (adjusted to OD_{600} = 0.5 in all cases), and oxygen consumption was measured during 90 s, plotting the values of oxygen saturation (μmol O_2) *versus* time (in min). The linear section of these plots were used to establish comparisons across strains and conditions as previously explained (Ruiz *et al.*, 2006).

Assessment of the total dehydrogenase activity as an indicator of overall metabolic vigor. RSG vitality staining was used to diagnose the metabolic state. The signal intensity of cells stained with the RSG reagent is altered when cells are treated with reagents that disrupt electron transport (e.g. CCCP, see below). In each set of experiments, 1×10^6 cells suspended in 0.5 ml of 50 mM phosphate buffer (pH = 7.5) were stained with 1 μl of the RSG solution provided by Life Technologies Corp. (USA). Staining procedures followed the instructions recommended by the manufacturer. In some experiments, cells were also stained with propidium iodide to evaluate vitality; bacteria with damaged cell membranes represented a very small population (<5%) during logarithmic growth in all the conditions tested. Negative controls were run by adding 2 μl of 5 mM CCCP to the cell suspension. The RSG fluorescence was quantified at 400 nm and 447 nm as the excitation and emission wavelengths, respectively, according to the protocol previously developed for *P. putida* (Nikel *et al.*, 2015b).

Statistical analysis. All reported experiments were independently repeated at least three times (as indicated in the figure legends), and mean values of the corresponding parameter and standard deviation is presented. The significance of differences when comparing results was evaluated by means of the Student's *t* test.

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F.C., J.L.M, and P.I.N. designed the project and the experimental layout. F.C. performed the experiments related and drafted the manuscript. F.C., J.L.M, and P.I.N. analyzed the data. J.L.M and P.I.N. wrote the manuscript. The authors wish to thank V. de Lorenzo (CNB-CSIC, Spain) for inspiring discussions.

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The authors declare that there are no competing interests associated with the contents of this article.

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TABLES

Table 1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'→3')	Description (reference)
*rplU*_F	CGCAGTGATTGTTACCGGTG	Checking for DNA contamination in RNA samples (Reales-Calderón *et al.*, 2015b)
*rplU*_R	AGGCCTGAATGCCGGTGATC	Checking for DNA contamination in RNA samples (Reales-Calderón *et al.*, 2015b)
*rpoN*_F	GCAGAAATACATGCATACG	Housekeeping gene used as internal control in RT-qPCR experiments (Reales-Calderón *et al.*, 2015b)
*rpoN*_R	TGTGCCTCCAGTAAACCAG	Housekeeping gene used as internal control in RT-qPCR experiments (Reales-Calderón *et al.*, 2015b)
*katA*_F	TCAAGTTCCCCGATCTCAAC	Gene *katA*, RT-qPCR
*katA*_R	GGATGTGCCGATAGCTCTTC	Gene *katA*, RT-qPCR
*katB*_F	CACCCTGACCCTGCTGTATT	Gene *katB*, RT-qPCR
*katB*_R	TTCGGATCGAGGTTCTTCTG	Gene *katB*, RT-qPCR
*sodB*_F	CCTTCGACAAGTTCAAGGAA	Gene *sodB*, RT-qPCR
*sodB*_R	GGTTGCGGTAGTCGATGTAG	Gene *sodB*, RT-qPCR
*sodM*_F	GACCATGGAGATCCACCAC	Gene *sodM*, RT-qPCR
*sodM*_R	GACATCACGGTCCAGAACA	Gene *sodM*, RT-qPCR
*ahpC*_F	TGTCCCTGATCAACACTCAA	Gene *ahpC*, RT-qPCR
*ahpC*_R	GGAACTCGCCGTAGTTGTT	Gene *ahpC*, RT-qPCR

FIGURE CAPTIONS

FIGURE 1. Mechanism of Crc-mediated post-transcriptional regulation in *P. aeruginosa*. In the presence of a preferred (primary) carbon source, the system is considered to be in an inactive state, in which both Crc and Hfq (an RNA chaperone) are bound to a specific sequence in the target mRNA (5'-AA*N*AA*N*AA-3', where *N* represents any nucleotide)—thus blocking the translation of the transcript. Upon consumption of the primary carbon source, a (yet unidentified) signal triggers the autophosphorylation of the CbrA sensor kinase, which in turn phosphorylates the CbrB response regulator (the phosphate residue is identified with a black circle in the diagram). These events ultimately lead to the expression of the small RNA CrcZ, which sequesters Crc and Hfq, enabling the translation of the target mRNA. Under this active state, a number of physiological and metabolic changes take place in the cells, including the activity of a number of specific regulators, enzymes, and transporters that are related to the utilization of the secondary carbon source.

FIGURE 2. Tolerance and response to oxidative stress in *P. aeruginosa*. (*A*) Oxidative stress susceptibility test. *P. aeruginosa* PAO1 (wild-type strain, WT) and its Δ*crc* derivative were seeded onto an LB medium agar plate and 1 μmol of either H_2O_2 or paraquat (PQ) was added onto each paper disk from a concentrated stock solution. The diameter of the growth inhibition halo (in mm) was measured after 20 h of incubation at 37°C. A representative image of the plates is shown with the average and standard deviations from three independent experiments. (*B*) *In vitro* determination of the total catalase (Kat) activity. The specific (Sp) Kat activity (represented by three different enzymes in *P. aeruginosa*) was assessed in cell-free extracts of the wild-type and Δ*crc* strains during the mid-exponential phase of growth in LB medium. The values are reported as the mean ± standard deviation from duplicate measurements from at least three independent experiments. A significant difference ($P < 0.05$, as evaluated by means of the Student's *t* test) in the pair-wise comparison of the Kat activity between the two strains is indicated by the * symbol.

FIGURE 3. Transcriptional analysis of genes involved in the oxidative stress response in *P. aeruginosa.* (*A*) The expression level of *katA*, *katB*, *sodB*, *sodM*, and *ahpC* was measured by means of RT-qPCR in LB medium cultures of *P. aeruginosa* Δ*crc* during the mid-exponential phase of growth. Results represent the mean value of three independent experiments from at least two reactions per sample, and the error bars indicate the standard deviation. The baseline (i.e. no changes in gene expression) is shown as a dashed grey line. (*B*) Expression of the same genes during an oxidative stress challenge in *P. aeruginosa* PAO1 (wild-type strain, WT) and its Δ*crc* derivative. In these experiments, LB medium cultures were added with H_2O_2 at 1 mM. Results represent the mean value of three independent experiments from at least two reactions per sample, and the error bars indicate the standard deviation. The baseline (i.e. no H_2O_2) is shown as a dashed grey line.

FIGURE 4. Respirometric and biochemical analyses of O_2 consumption and ROS formation in *P. aeruginosa*. (*A*) Specific rates of O_2 consumption in LB medium cultures during the mid-exponential phase of growth. Respiration rates were measured in the two strains using an oximeter based on a Clark-type electrode, and the average values of respiration rates in the Δ*crc* mutant were normalized by comparison to those in the wild-type strain (WT = 1, baseline indicated with a dashed grey line). (*B*) Superoxide anion ($O_2{\cdot}^-$) formation in LB medium cultures during the mid-exponential phase of growth. A biochemical method, based on the reduction of nitro-blue tetrazolium chloride (NBT), was used to estimate the formation of ROS in both the WT strain and the Δ*crc* mutant. To induce the production of $O_2{\cdot}^-$ (positive control), paraquat (PQ) was added at 1 mM and the $O_2{\cdot}^-$ formation was measured after 30 min. The average values of NBT reduction in the assays are represented as a fraction of the values obtained for the WT strain in the absence of PQ (control experiment, Ctrl.). (*C*) Total metabolic activity as estimated by means of the chemical sensor *Bac*Light™ RedoxSensor™ Green (RSG). Cells were sampled from LB medium cultures during the mid-exponential phase of growth, and the RSF fluorescence was normalized to the cell density in each sample (R.F.U., relative fluorescence units). In some of the experiments, the ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to the culture medium at 10 μM. In all the experiments shown in this figure, the values are reported as the mean ± standard deviation from duplicate measurements from at least three independent

experiments. A significant difference ($P < 0.05$, as evaluated by means of the Student's *t* test) in the pair-wise comparison of each parameter between the two strains is indicated by the * symbol.

FIGURE 5. Transcriptional and post-transcriptional control exerted by Crc on central carbon metabolism in *P. aeruginosa*. Simplified representation of the central carbon metabolism in *P. aeruginosa* PAO1 as deduced from the *Pseudomonas* database (Winsor *et al.*, 2016) and information from the literature (Bartell *et al.*, 2017). Changes in the transcriptome (*T*) and proteome (*P*) when comparing the Δ*crc* mutant *versus* the wild-type strain in exponential cultures in LB medium are shown for specific nodes in the network using colored dots. In all cases, the first dot indicates variations in mRNA levels and the second dot identifies changes in the protein level for each reaction according to the color code shown in the upper right corner of the scheme. Reactions that had no changes (NC) among experimental conditions or that were not detected (ND) are likewise indicated. The number of rows indicate the number of orthologues annotated to catalyze a given metabolic step (e.g. FumC1, FumC2, and PA_4333, all mediating the hydration of fumarate into malate, are identified by three rows of dots—in each row, the first dot shows changes in the transcriptome and the second dot shows changes in the proteome). The main metabolic blocks in central carbon metabolism are identified by means of different colors, and the reactions within these blocks are numbered [**1**, glucokinase; **2**, glucose dehydrogenase; **3**, glucose-6-*P* dehydrogenase; **4**, 6-phosphogluconate dehydratase; **5**, 2-dehydro-3-deoxygluconate-6-*P* (KDPG) aldolase; **6**, glyceraldehyde-3-*P* dehydrogenase; **7**, pyruvate dehydrogenase; **8**, fumarate hydratase; **9**, malate dehydrogenase; and **10**, oxaloacetate decarboxylase]. Abbreviations are as follows: EMP pathway, Embden-Meyerhof-Parnas pathway; ED pathway, Entner-Doudoroff pathway; PP pathway, pentose phosphate pathway; TCA cycle, tricarboxylic acid cycle; F6P, fructose-6-*P*; FBP, fructose-1,6-P_2; G6P, glucose-6-*P*; 6PG, 6-phosphogluconate; 2KG, 2-ketogluconate; 2K6PG, 2-keto-6-phosphogluconate; PEP, phosphoenolpyruvate; Pyr, pyruvate; G3P, glyceraldehyde-3-*P*; BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; DHAP, dihydroxyacetone-*P*; OAA, oxaloacetate; AcCoA, acetyl-coenzyme A; SucCoA, succinyl-coenzyme A; and αKG, α-ketoglutarate.

FIGURE 6. *In vitro* determination of key enzymatic activities regulated by Crc in *P. aeruginosa*. (A) Specific (Sp) Edd/Eda activities [6-phosphogluconate (6PG) dehydratase and 2-dehydro-3-deoxygluconate-6-*P* aldolase, respectively], which yield pyruvate (Pyr) and glyceraldehyde-3-*P* (GA3P). (*B*) and (*C*) Specific Zwf (glucose-6-*P* dehydrogenase) activity, which transforms glucose-6-*P* (G6P) into 6-phoshogluconate (6PG) in a $NADP^+$-dependent reaction (note that the transformation catalyzed by Pgl is implicit in this reaction). In all cases, cell-free extracts were prepared from *P. aeruginosa* PA01 (wild-type strain, WT) and its Δ*crc* derivative harvested from LB medium cultures during the mid-exponential phase of growth. In some experiments, paraquat (PQ) was added at 1 mM as an oxidative stress agent. The gray dashed line indicates the baseline of Zwf activity in the WT strain in the absence of PQ (control experiment, Ctrl.). In all the experiments shown, the values are reported as the mean ± standard deviation from four measurements from at least three independent experiments. A significant difference ($P < 0.05$, as evaluated by means of the Student's *t* test) in the pair-wise comparison of each enzymatic activity between the two strains is indicated by the * symbol.

FIGURE 7. Redox balances of pyridine nucleotide cofactors. Total and individual redox ratios were determined from the absolute intracellular concentrations of NAD^+, NADH, $NADP^+$, and NADPH. All pyridine nucleotide cofactors were determined enzymatically in exponentially-growing *P. aeruginosa* PAO1 (wild-type strain, WT) and its Δ*crc* derivative in LB medium. In all cases, each bar represents the mean value of the corresponding redox ratio ± standard deviation of duplicate measurements from at least two independent experiments. A significant difference ($P < 0.05$, as evaluated by means of the Student's *t* test) in the pair-wise comparison of each redox ratio between the two strains is indicated by the * symbol.