

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

Corona, Fernando; Martínez, José Luis; Nikel, Pablo I.

Published in: Environmental Microbiology

Link to article, DOI: 10.1111/1462-2920.14471

Publication date: 2019

Document Version Peer reviewed version

Link back to DTU Orbit

Citation (APA): Corona, F., Martínez, J. L., & Nikel, P. I. (2019). The global regulator Crc orchestrates the metabolic robustness underlying oxidative stress resistance in *Pseudomonas aeruginosa*. *Environmental Microbiology*, *21*(3), 898-912. https://doi.org/10.1111/1462-2920.14471

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.

- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

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

by

Fernando Corona¹, José Luis Martínez^{1*}, and Pablo I. Nikel^{2*}

- ¹ Department of Microbial Biotechnology, Centro Nacional de Biotecnología (CNB-CSIC), 28049 Madrid, Spain
- ² Systems Environmental Microbiology Group, The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2800 Kgs Lyngby, Denmark

Running title:	Crc mediates stress resistance in <i>P. aeruginosa</i>		
Keywords:	Pseudomonas aeruginosa · Crc · Metabolism · Stress · Regulation ·		
	Bacterial physiology		

Correspondence to:	Pablo I. Nikel (pabnik@biosustain.dtu.dk)	
	The Novo Nordisk Foundation Center for Biosustainability,	
	Technical University of Denmark	
	2800 Lyngby, Denmark	
	Tel: (+45 93) 51 19 18	
	José L. Martínez (jlmtnez@cnb.csic.es)	
	Centro Nacional de Biotecnología (CNB-CSIC)	
	28049 Madrid, Spain	
	Tel: (+34 91) 585 4542	

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1462-2920.14471

This article is protected by copyright. All rights reserved.

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 *\(\Delta\)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 O₂ (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 Hfg 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/Hfg 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* Δcrc 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 Δcrc 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 Δcrc 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 (H₂O₂) or paraquat (PQ, *N*,*N*-dimethyl-4,4'-bipyridinium dichloride, an oxidative agent that generates superoxide, O₂.-) were measured and compared (**Fig. 2A**). The Δcrc 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 H₂O₂ or 1 µmol of PQ increased by 1.3- and 1.5-fold, respectively, when comparing the Δcrc mutant with the wild-type strain.

Considering the increased susceptibility of the Δcrc 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 Δcrc mutant grown in LB medium (**Fig. 2B**). Consistent with the high susceptibility to oxidative stress observed in the Δcrc 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 $\triangle crc$ 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 $\triangle crc$ mutant when compared to the wild-type strain. However, the expression of *katB* has a 3-fold increase in the $\triangle crc$ mutant. The expression of *katB* has been

Chailenge (Ochsher et al., 2000). Since katz during aerobic growth, this mutant seems to oxidative stress than the parental strain—thut total Kat activity. Furthermore, examination of mediated regulation in *P. aeruginosa* PAO1 strain undergoes a basal level of oxidative strain slight (but significant) increase in protein abur The level of expression of key genes encoder response to an oxidative stress challenge with derivative were grown in LB medium as indice of these cultures. The expression of *katA*, *ka* presence of H₂O₂ in the wild-type strain (Fig. that ranged from 52-fold (*ahpC*) up to 491-for

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 Δcrc 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 Δcrc 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 log₂($\Delta crc/PAO1$) > 0.3].

The level of expression of key genes encoding ROS defense functions was also measured in response to an oxidative stress challenge with H₂O₂. To this end, the wild-type strain and its Δcrc derivative were grown in LB medium as indicated above, and H₂O₂ 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 H₂O₂ 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 Δcrc strain by treatment with H₂O₂; 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 Δcrc 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 Δcrc mutant. In all, the exploration of the transcriptional landscape of these strains by RT-qPCR revealed that the Δcrc 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 Δcrc 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 -- 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 $\triangle crc$ mutant as compared to the wild-type strain (**Fig. 4A**). Such significant increase in O₂ consumption in the mutant strain could correlate to enhanced O₂.- formation, and a biochemical test was adopted to quantify O₂.- by means of the redox-sensitive compound nitroblue tetrazolium chloride (NBT). To this end, NBT reduction was quantified in both *P. aeruginosa* PAO1 and its $\triangle crc$ derivative under the same growth conditions indicated above (**Fig. 4B**). The formation of O₂.- in this experiment followed the same trend as the specific rates of O₂ consumption, i.e. the $\triangle crc$ 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 O₂.- formation in the two strains—yet, the $\triangle crc$ mutant had a larger increase in ROS accumulation upon exposure to PQ. Interestingly, the $\triangle crc$ mutant growing in the absence of any stressor showed levels of O₂.- 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 Δcrc 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 BacLight™ 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 Δcrc 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 Δcrc 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 -- and NADH oxidation, redoxactive 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 Δcrc 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 Δcrc mutant is subjected to a basal level of elevated stress due to increased respiration and O₂ - 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 Δcrc mutant against the wild-type strain. We gathered data both from RNA sequencing and isobaric tags for relative and absolute quantitation (/TRAQ)-based proteomics (Reales-Calderón et al., 2015b; Corona et al., 2018) in exponentiallygrowing LB medium cultures, in which the Crc-dependent catabolite repression is maximal (Valentini *et al.*, 2014), for *P. aeruginosa* PA01 and its Δcrc 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* \triangle *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 log₂($\Delta crc/PAO1$) < -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 Δcrc 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) \rightarrow Zwf [first step of the pentose phosphate (PP) pathway] \rightarrow Edd/Eda (catabolic Entner-Doudoroff pathway). This pattern of catabolic activities suggests a role of the upper domain of the biochemical network in the Δcrc 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 Δcrc 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-3deoxygluconate-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 $\triangle crc$ 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 Δcrc 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 Δcrc 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 Δcrc 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 Δcrc 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 Δcrc mutant strains (**Fig. 7**). The NADPH/NADP⁺ ratio, in contrast, was significantly higher (1.8fold) in the Δcrc 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 Δcrc 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 Δcrc 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 .-, 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 $\triangle crc$ mutant, and this strain is considerably more susceptible to oxidative stress agents than its parental counterpart

(Fig. 2). Secondly, in such conditions, the Δcrc strain had higher levels of O_2 - 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 Δcrc 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, lcd (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 lcd activity was assayed in the strains under study, and a small decrease could be detected in the $\triangle crc$ 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 Δcrc 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 Δcrc 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 \rightarrow 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 $\triangle crc$ strain, as previously suggested by Price-Whelan *et al.* (2007).

In summary, the physiological situation characteristic of the Δcrc 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 wildtype strain *P. aeruginosa* PAO1 (Holloway, 1969), a generous gift of P. V. Phibbs, and its isogenic, marker-less Δcrc 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 (OD₆₀₀) of 0.05. Quantitative physiology experiments and transcriptional analysis were carried out with cells harvested at the mid-exponential phase of growth (when OD₆₀₀ = 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 N₂, 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 H₂O₂ 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 ($5,800 \times g$, 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 inbetween 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 \cdot 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 μ I of the cell-free extracts with 1 ml of 10% (vol/vol) H₂O₂ 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 H₂O₂ decomposition, and a molar extinction coefficient ε = 43.6 M⁻¹ · 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 μ l of the following reaction mixtures (all concentrations indicated are final): (i) Zwf activity; 50 mM phosphate buffer (pH = 7.5), 10 mM MgSO₄, 0.75 mM NADP⁺, and 2 mM glucose-6-*P*; (ii) Icd activity; 67 mM phosphate buffer (pH = 7.5), 0.6 mM MnCl₂, 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 ϵ = 6.22 mM⁻¹ · 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·HCI (pH = 7.5), 10 mM MgCl₂, 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·HCI (pH = 7.5), 10 mM MgCl₂, 0.1 mM NADH, and 0.5 U of L-lactate dehydrogenase (RocheTM 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 · 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 aliguots were promptly harvested from midexponential 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 N₂. Three hundred microliters of 0.2 M NaOH [for NAD(P)H extraction] or 300 µl of 0.2 M HCI [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 $(12,000 \times g, 5 \text{ min}, 4^{\circ}\text{C})$. Five microliters of the resulting supernatants were mixed with 90 μ l of a reaction mixture containing (final concentrations in a final volume of 100 μ l): 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 · ml-1 [for NAD(+/H) determinations] or glucose-6-P dehydrogenase (Sigma-Aldrich Co.) at 5 U · 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 (A₅₇₀) 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 ($12,000 \times g$, 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 μ g · 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 μ l 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 (OD₆₀₀) 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.

ACKNOWLEDGEMENTS

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.

This study was supported by The Novo Nordisk Foundation (Grant NNF10CC1016517) and the Danish Council for Independent Research (*SWEET*, DFF-Research Project 8021-00039B) to P.I.N. This work was also supported by grants from the Instituto de Salud Carlos III (Spanish Network for Research on Infectious Diseases [RD16/0016/0011]), from the Spanish Ministry of

Economy and Competitiveness (BIO2017-83128-R), from CNB and from the Autonomous Community of Madrid (B2017/BMD-3691) to J.L.M.

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

- Arce-Rodríguez, A., Calles, B., Nikel, P.I., and de Lorenzo, V. (2016) The RNA chaperone Hfq enables the environmental stress tolerance super-phenotype of *Pseudomonas putida*. *Environ Microbiol* **18**: 3309-3326.
- Balasubramanian, D., Kumari, H., and Mathee, K. (2015) *Pseudomonas aeruginosa* AmpR: An acute-chronic switch regulator. *Pathog Dis* **73**: 1-14.
- Bartell, J.A., Blazier, A.S., Yen, P., Thøgersen, J.C., Jelsbak, L., Goldberg, J.B., and Papin, J.A. (2017) Reconstruction of the metabolic network of *Pseudomonas aeruginosa* to interrogate virulence factor synthesis. *Nat Commun* 8: 14631.
- Belda, E., van Heck, R.G.A., López-Sánchez, M.J., Cruveiller, S., Barbe, V., Fraser, C. *et al.* (2016) The revisited genome of *Pseudomonas putida* KT2440 enlightens its value as a robust metabolic *chassis*. *Environ Microbiol* **18**: 3403-3424.
- Benedetti, I., de Lorenzo, V., and Nikel, P.I. (2016) Genetic programming of catalytic *Pseudomonas putida* biofilms for boosting biodegradation of haloalkanes. *Metab Eng* **33**: 109-118.
- Bernofsky, C., and Swan, M. (1973) An improved cycling assay for nicotinamide adenine dinucleotide. *Anal Biochem* **53**: 452-458.
- Brown, S.M., Howell, M.L., Vasil, M.L., Anderson, A.J., and Hassett, D.J. (1995) Cloning and characterization of the *katB* gene of *Pseudomonas aeruginosa* encoding a hydrogen peroxide-inducible catalase: purification of KatB, cellular localization, and demonstration that it is essential for optimal resistance to hydrogen peroxide. *J Bacteriol* **177**: 6536-6544.
- Chavarría, M., Nikel, P.I., Pérez-Pantoja, D., and de Lorenzo, V. (2013) The Entner-Doudoroff pathway empowers *Pseudomonas putida* KT2440 with a high tolerance to oxidative stress. *Environ Microbiol* **15**: 1772-1785.
- Collier, D.N., Hager, P.W., and Phibbs, P.V. (1996) Catabolite repression control in the Pseudomonads. *Res Microbiol* **147**: 551-561.
- Corona, F., Reales-Calderón, J.A., Gil, C., and Martínez, J.L. (2018) The development of a new parameter for tracking posttranscriptional regulation allows the detailed map of the *Pseudomonas aeruginosa* Crc regulon. *Sci Rep*: In press, DOI: 10.1038/s41598-41018-34741-41599.

- Decorosi, F., Lori, L., Santopolo, L., Tatti, E., Giovannetti, L., and Viti, C. (2011) Characterization of a Cr(VI)-sensitive *Pseudomonas corrugata* 28 mutant impaired in a pyridine nucleotide transhydrogenase gene. *Res Microbiol* **162**: 747-755.
- Deutsch, E.W., Csordas, A., Sun, Z., Jarnuczak, A., Perez-Riverol, Y., Ternent, T. *et al.* (2017) The *ProteomeXchange* consortium in 2017: supporting the cultural change in proteomics public data deposition. *Nucleic Acids Res* **45**: D1100-D1106.
- Dvořák, P., Chrást, L., Nikel, P.I., Fedr, R., Soucek, K., Sedlacková, M. et al. (2015) Exacerbation of substrate toxicity by IPTG in *Escherichia coli* BL21(DE3) carrying a synthetic metabolic pathway. *Microb Cell Fact* 14: 201.
- Esterhazy, D., King, M.S., Yakovlev, G., and Hirst, J. (2008) Production of reactive oxygen species by complex I (NADH:ubiquinone oxidoreductase) from *Escherichia coli* and comparison to the enzyme from mitochondria. *Biochemistry* **47**: 3964-3971.
- Folkesson, A., Jelsbak, L., Yang, L., Johansen, H.K., Ciofu, O., Høiby, N., and Molin, S. (2012) Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. *Nat Rev Microbiol* **10**: 841-851.
- Fuhrer, T., and Sauer, U. (2009) Different biochemical mechanisms ensure network-wide balancing of reducing equivalents in microbial metabolism. *J Bacteriol* **191**: 2112-2121.
- Grenga, L., Little, R.H., and Malone, J.G. (2017) Quick change: post-transcriptional regulation in *Pseudomonas*. *FEMS Microbiol Lett* **364**: fnx125.
- Hare, N.J., Scott, N.E., Shin, E.H., Connolly, A.M., Larsen, M.R., Palmisano, G., and Cordwell, S.J. (2011) Proteomics of the oxidative stress response induced by hydrogen peroxide and paraquat reveals a novel AhpC-like protein in *Pseudomonas aeruginosa*. *Proteomics* **11**: 3056-3069.
- Hassett, D.J., Howell, M.L., Ochsner, U.A., Vasil, M.L., Johnson, Z., and Dean, G.E. (1997) An operon containing *fumC* and *sodA* encoding fumarase C and manganese superoxide dismutase is controlled by the ferric uptake regulator in *Pseudomonas aeruginosa: fur* mutants produce elevated alginate levels. *J Bacteriol* **179**: 1452-1459.
- Holloway, B.W. (1969) Genetics of Pseudomonas. Bacteriol Rev 33: 419-443.
- Imlay, J.A. (2013) The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat Rev Microbiol* **11**: 443-454.

- Kadenbach, B. (2003) Intrinsic and extrinsic uncoupling of oxidative phosphorylation. *Biochim Biophys Acta* **1604**: 77-94.
- Kalyuzhnaya, M.G., Lidstrom, M.E., and Chistoserdova, L. (2008) Real-time detection of actively metabolizing microbes by redox sensing as applied to methylotroph populations in Lake Washington. *ISME J* 2: 696-706.
- Kambara, T.K., Ramsey, K.M., and Dove, S.L. (2018) Pervasive targeting of nascent transcripts by Hfq. Cell Rep 23: 1543-1552.
- Kim, J., Jeon, C.O., and Park, W. (2008) Dual regulation of *zwf-1* by both 2-keto-3-deoxy-6phosphogluconate and oxidative stress in *Pseudomonas putida*. *Microbiology* **154**: 3905-3916.
- Kim, J., Oliveros, J.C., Nikel, P.I., de Lorenzo, V., and Silva-Rocha, R. (2013) Transcriptomic fingerprinting of *Pseudomonas putida* under alternative physiological regimes. *Environ Microbiol Rep* **5**: 883-891.
- Kim, J., and Park, W. (2014) Oxidative stress response in *Pseudomonas putida*. Appl Microbiol Biotechnol **98**: 6933-6946.
- La Rosa, R., Nogales, J., and Rojo, F. (2015) The Crc/CrcZ-CrcY global regulatory system helps the integration of gluconeogenic and glycolytic metabolism in *Pseudomonas putida*. *Environ Microbiol* **17**: 3362-3378.
- La Rosa, R., Behrends, V., Williams, H.D., Bundy, J.G., and Rojo, F. (2016) Influence of the Crc regulator on the hierarchical use of carbon sources from a complete medium in *Pseudomonas. Environ Microbiol* **18**: 807-818.
- La Rosa, R., Johansen, H.K., and Molin, S. (2018) Convergent metabolic specialization through distinct evolutionary paths in *Pseudomonas aeruginosa. mBio* **9**: e00269-00218.
- Lamprecht, D.A., Finin, P.M., Rahman, M.A., Cumming, B.M., Russell, S.L., Jonnala, S.R. *et al.* (2016) Turning the respiratory flexibility of *Mycobacterium tuberculosis* against itself. *Nat Commun* **7**: 12393.
- Lau, G.W., Hassett, D.J., Ran, H., and Kong, F. (2004) The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends Mol Med* **10**: 599-606.
- Lemire, J., Alhasawi, A., Appanna, V.P., Tharmalingam, S., and Appanna, V.D. (2017) Metabolic defence against oxidative stress: the road less travelled so far. *J Appl Microbiol* **123**: 798-809.

- Linares, J.F., Moreno, R., Fajardo, A., Martínez-Solano, L., Escalante, R., Rojo, F., and Martínez, J.L. (2010) The global regulator Crc modulates metabolism, susceptibility to antibiotics and virulence in *Pseudomonas aeruginosa*. *Environ Microbiol* **12**: 3196-3212.
- Liu, Y., Gokhale, C.S., Rainey, P.B., and Zhang, X.X. (2017) Unravelling the complexity and redundancy of carbon catabolic repression in *Pseudomonas fluorescens* SBW25. *Mol Microbiol* **105**: 589-605.
- Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time guantitative PCR and the 2-DDCT method. *Methods* **25**: 402-408.
- Lu, J., and Holmgren, A. (2014) The thioredoxin superfamily in oxidative protein folding. *Antioxid Redox Signal* **21**: 457-470.
- Ma, J.F., Hager, P.W., Howell, M.L., Phibbs, P.V., and Hassett, D.J. (1998) Cloning and characterization of the *Pseudomonas aeruginosa zwf* gene encoding glucose-6-phosphate dehydrogenase, an enzyme important in resistance to methyl viologen (paraquat). *J Bacteriol* **180**: 1741-1749.
- Mailloux, R.J., Lemire, J., and Appanna, V.D. (2011) Metabolic networks to combat oxidative stress in *Pseudomonas fluorescens*. *Antonie van Leeuwenhoek* **99**: 433-442.
- Maklashina, E., Rajagukguk, S., Iverson, T.M., and Cecchini, G. (2018) The unassembled flavoprotein subunits of human and bacterial complex II have impaired catalytic activity and generate only minor amounts of ROS. *J Biol Chem* **293**: 7754-7765.
- Milojevic, T., Grishkovskaya, I., Sonnleitner, E., Djinovic-Carugo, K., and Bläsi, U. (2013) The *Pseudomonas aeruginosa* catabolite repression control protein Crc is devoid of RNA binding activity. *PLoS One* **8**: e64609.
- Moradali, M.F., Ghods, S., and Rehm, B.H.A. (2017) *Pseudomonas aeruginosa* lifestyle: A paradigm for adaptation, survival, and persistence. *Front Cell Infect Microbiol* **7**: 39.
- Moreno, R., Marzi, S., Romby, P., and Rojo, F. (2009) The Crc global regulator binds to an unpaired A-rich motif at the *Pseudomonas putida alkS* mRNA coding sequence and inhibits translation initiation. *Nucleic Acids Res* **37**: 7678-7690.
- Moreno, R., Hernández-Arranz, S., La Rosa, R., Yuste, L., Madhushani, A., Shingler, V., and Rojo, F. (2015) The Crc and Hfq proteins of *Pseudomonas putida* cooperate in catabolite repression and formation of ribonucleic acid complexes with specific target motifs. *Environ Microbiol* **17**: 105-118.

- Nikel, P.I., Giordano, A.M., de Almeida, A., Godoy, M.S., and Pettinari, M.J. (2010) Elimination of D-lactate synthesis increases poly(3-hydroxybutyrate) and ethanol synthesis from glycerol and affects cofactor distribution in recombinant *Escherichia coli*. *Appl Environ Microbiol* **76**: 7400-7406.
- Nikel, P.I., Chavarría, M., Martínez-García, E., Taylor, A.C., and de Lorenzo, V. (2013) Accumulation of inorganic polyphosphate enables stress endurance and catalytic vigour in *Pseudomonas putida* KT2440. *Microb Cell Fact* **12**: 50.
- Nikel, P.I., Kim, J., and de Lorenzo, V. (2014) Metabolic and regulatory rearrangements underlying glycerol metabolism in *Pseudomonas putida* KT2440. *Environ Microbiol* 16: 239-254.
- Nikel, P.I., Chavarría, M., Fuhrer, T., Sauer, U., and de Lorenzo, V. (2015a) *Pseudomonas putida* KT2440 strain metabolizes glucose through a cycle formed by enzymes of the Entner-Doudoroff, Embden-Meyerhof-Parnas, and pentose phosphate pathways. *J Biol Chem* **290**: 25920-25932.
- Nikel, P.I., Romero-Campero, F.J., Zeidman, J.A., Goñi-Moreno, A., and de Lorenzo, V. (2015b) The glycerol-dependent metabolic persistence of *Pseudomonas putida* KT2440 reflects the regulatory logic of the GlpR repressor. *mBio* 6: e00340-00315.
- Nikel, P.I., and Chavarría, M. (2016) Quantitative physiology approaches to understand and optimize reducing power availability in environmental bacteria. In *Hydrocarbon and Lipid Microbiology Protocols–Synthetic and Systems Biology - Tools*. McGenity, T.J., Timmis, K.N., and Nogales-Fernández, B. (eds). Heidelberg, Germany: Humana Press, pp. 39-70.
- Nikel, P.I., Pérez-Pantoja, D., and de Lorenzo, V. (2016) Pyridine nucleotide transhydrogenases enable redox balance of *Pseudomonas putida* during biodegradation of aromatic compounds. *Environ Microbiol* **18**: 3565-3582.
- O'Toole, G.A., Gibbs, K.A., Hager, P.W., Phibbs, P.V., and Kolter, R. (2000) The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. *J Bacteriol* **182**: 425-431.
- Ochsner, U.A., Vasil, M.L., Alsabbagh, E., Parvatiyar, K., and Hassett, D.J. (2000) Role of the *Pseudomonas aeruginosa oxyR-recG* operon in oxidative stress defense and DNA repair: OxyR-dependent regulation of *katB-ankB*, *ahpB*, and *ahpC-ahpF*. *J Bacteriol* **182**: 4533-4544.

- Palmer, K.L., Aye, L.M., and Whiteley, M. (2007) Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. *J Bacteriol* **189**: 8079-8087.
- Pérez-Pantoja, D., Nikel, P.I., Chavarría, M., and de Lorenzo, V. (2013) Endogenous stress caused by faulty oxidation reactions fosters evolution of 2,4-dinitrotoluene-degrading bacteria. *PLoS Genet* **9**: e1003764.
- Price-Whelan, A., Dietrich, L.E., and Newman, D.K. (2007) Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14. J *Bacteriol* 189: 6372-6381.
- Rahme, L.G., Ausubel, F.M., Cao, H., Drenkard, E., Goumnerov, B.C., Lau, G.W. et al. (2000) Plants and animals share functionally common bacterial virulence factors. *Proc Natl Acad Sci USA* 97: 8815-8821.
- Reales-Calderón, J.A., Corona, F., Monteoliva, L., Gil, C., and Martínez, J.L. (2015a) Quantitative proteomics unravels that the post-transcriptional regulator Crc modulates the generation of vesicles and secreted virulence determinants of *Pseudomonas aeruginosa*. *Data in Brief* **4**: 450-453.
- Reales-Calderón, J.A., Corona, F., Monteoliva, L., Gil, C., and Martínez, J.L. (2015b) Quantitative proteomics unravels that the post-transcriptional regulator Crc modulates the generation of vesicles and secreted virulence determinants of *Pseudomonas aeruginosa*. *J Proteomics* **127**: 352-364.
- Rojo, F. (2010) Carbon catabolite repression in *Pseudomonas*: optimizing metabolic versatility and interactions with the environment. *FEMS Microbiol Rev* **34**: 658-684.
- Ruiz, J.A., Fernández, R.O., Nikel, P.I., Méndez, B.S., and Pettinari, M.J. (2006) *dye* (*arc*) Mutants: insights into an unexplained phenotype and its suppression by the synthesis of poly(3-hydroxybutyrate) in *Escherichia coli* recombinants. *FEMS Microbiol Lett* **258**: 55-60.
- Sabra, W., Lünsdorf, H., and Zeng, A.P. (2003) Alterations in the formation of lipopolysaccharide and membrane vesicles on the surface of *Pseudomonas aeruginosa* PAO1 under oxygen stress conditions. *Microbiology* 149: 2789-2795.
- Sánchez, P., Linares, J.F., Ruiz-Díez, B., Campanario, E., Navas, A., Baquero, F., and Martínez, J.L. (2002) Fitness of *in vitro* selected *Pseudomonas aeruginosa nalB* and *nfxB* multidrug resistant mutants. *J Antimicrob Chemother* **50**: 657-664.

- Sandoval, J.M., Arenas, F.A., and Vásquez, C.C. (2011) Glucose-6-phosphate dehydrogenase protects *Escherichia coli* from tellurite-mediated oxidative stress. *PLoS One* **6**: e25573.
- Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012) NIH Image to *ImageJ*: 25 years of image analysis. *Nat Methods* **9**: 671-675.
- Scialò, F., Fernández-Ayala, D.J., and Sanz, A. (2017) Role of mitochondrial reverse electron transport in ROS signaling: potential roles in health and disease. *Front Physiol* **8**: 428.
- Shin, D.H., Choi, Y.S., and Cho, Y.H. (2008) Unusual properties of catalase A (KatA) of *Pseudomonas aeruginosa* PA14 are associated with its biofilm peroxide resistance. J *Bacteriol* **190**: 2663-2670.
- Spaans, S.K., Weusthuis, R.A., van der Oost, J., and Kengen, S.W. (2015) NADPH-generating systems in bacteria and archaea. *Front Microbiol* **6**: 742.
- Tribelli, P.M., Nikel, P.I., Oppezzo, O.J., and López, N.I. (2013) Anr, the anaerobic global regulator, modulates the redox state and oxidative stress resistance in *Pseudomonas extremaustralis*. *Microbiology* **159**: 259-268.
- Turner, J.M., and Messenger, A.J. (1986) Occurrence, biochemistry and physiology of phenazine pigment production. *Adv Microb Physiol* **27**: 211-275.
- Udaondo, Z., Ramos, J.L., Segura, A., Krell, T., and Daddaoua, A. (2018) Regulation of carbohydrate degradation pathways in *Pseudomonas* involves a versatile set of transcriptional regulators. *Microb Biotechnol* **11**: 442-454.
- Valentini, M., García-Mauriño, S.M., Pérez-Martínez, I., Santero, E., Canosa, I., and Lapouge, K. (2014) Hierarchical management of carbon sources is regulated similarly by the CbrA/B systems in *Pseudomonas aeruginosa* and *Pseudomonas putida*. *Microbiology* **160**: 2243-2252.
- Velázquez, F., di Bartolo, I., and de Lorenzo, V. (2004) Genetic evidence that catabolites of the Entner-Doudoroff pathway signal C source repression of the s⁵⁴ Pu promoter of Pseudomonas putida. J Bacteriol **186**: 8267-8275.
- Vizcaíno, J.A., Csordas, A., del Toro, N., Dianes, J.A., Griss, J., Lavidas, I. *et al.* (2016) 2016 update of the *PRIDE* database and its related tools. *Nucleic Acids Res* **44**: D447-D456.
- Wei, Q., Minh, P.N., Dotsch, A., Hildebrand, F., Panmanee, W., Elfarash, A. *et al.* (2012) Global regulation of gene expression by OxyR in an important human opportunistic pathogen. *Nucleic Acids Res* **40**: 4320-4333.

- Wilkes, R.A., Mendonca, C.M., and Aristilde, L. (2018) A cyclic metabolic network in *Pseudomonas protegens* Pf-5 prioritizes the Entner-Doudoroff pathway and exhibits substrate hierarchy during carbohydrate co-utilization. *Appl Environ Microbiol*: In press, DOI: 10.1128/AEM.02084-02018.
- Winsor, G.L., Griffiths, E.J., Lo, R., Dhillon, B.K., Shay, J.A., and Brinkman, F.S. (2016) Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* Genome Database. *Nucleic Acids Res* 44: D646-D653.
- Wirebrand, L., Madhushani, A.W.K., Irie, Y., and Shingler, V. (2018) Multiple Hfq-Crc target sites are required to impose catabolite repression on (methyl)phenol metabolism in *Pseudomonas putida* CF600. *Environ Microbiol* **20**: 186-199.
- Wolff, J.A., MacGregor, C.H., Eisenberg, R.C., and Phibbs, P.V. (1991) Isolation and characterization of catabolite repression control mutants of *Pseudomonas aeruginosa* PAO. *J Bacteriol* **173**: 4700-4706.
- Ying, W. (2008) NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation and biological consequences. *Antioxid Redox Signal* **10**: 179-206.

Oligonucleotide	Sequence (5'→3')	Description (reference)
<i>rpIU_</i> F	CGCAGTGATTGTTACCGGTG	Checking for DNA contamination in
		RNA samples (Reales-Calderón et al.,
		2015b)
rpIU_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)
<i>kat</i> A_F	TCAAGTTCCCCGATCTCAAC	Gene <i>kat</i> A, RT-qPCR
<i>kat</i> A_R	GGATGTGCCGATAGCTCTTC	Gene <i>kat</i> A, RT-qPCR
<i>katB_</i> F	CACCCTGACCCTGCTGTATT	Gene <i>katB</i> , RT-qPCR
<i>katB_</i> R	TTCGGATCGAGGTTCTTCTG	Gene <i>katB</i> , 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
ahnC R	GGAACTCGCCGTAGTTGTT	Gene ahpC RT-gPCR

 Table 1. Oligonucleotides used in this study.

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'-AANAANAA-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 $\triangle crc$ derivative were seeded onto an LB medium agar plate and 1 µmol of either H₂O₂ 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 $\triangle crc$ 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* Δcrc 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 Δcrc derivative. In these experiments, LB medium cultures were added with H₂O₂ 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 H₂O₂) is shown as a dashed grey line.

FIGURE 4. Respirometric and biochemical analyses of O₂ consumption and ROS formation in P. aeruginosa. (A) Specific rates of O₂ consumption in LB medium cultures during the midexponential 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 Δcrc 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 ·-) 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 Δcrc mutant. To induce the production of O₂ - (positive control), paraguat (PQ) was added at 1 mM and the O2-- 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 BacLight[™] 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 Δcrc 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, 2dehydro-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-P₂; G6P, glucose-6-P; 6PG, 6phosphogluconate; 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 α KG, 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 Δcrc 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 Δcrc 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.





This article is protected by copyright. All rights reserved.











This article is protected by copyright. All rights reserved.