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The acetoin assimilation pathway of *Pseudomonas putida* KT2440 is regulated by overlapping global regulatory elements that respond to nutritional cues

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Abstract

Many microorganisms produce and excrete acetoin (3-hydroxy-2-butanone) when growing in environments that contain glucose or other fermentable carbon sources. This excreted compound can then be assimilated by other bacterial species such as pseudomonads. This work shows that acetoin is not a preferred carbon source of *Pseudomonas putida*, and that the induction of genes required for its assimilation is down-modulated by different, independent, global regulatory systems when succinate, glucose or components of the LB medium are also present. The expression of the acetoin degradation genes was found to rely on the RpoN alternative sigma factor and to be modulated by the Crc/Hfq, Cyo and PTS^{Ntr} regulatory elements, with the impact of the latter three varying according to the carbon source present in addition to acetoin. Pyruvate, a poor carbon source for *P. putida*, did not repress acetoin assimilation. Indeed, the presence of acetoin significantly improved growth on pyruvate, revealing these compounds to have a synergistic effect. This would provide a clear competitive advantage to *P. putida* when growing in environments in which all the preferred carbon sources have been depleted and pyruvate and acetoin remain as leftovers from the fermentation of sugars by other microorganisms.

INTRODUCTION

When different bacterial species coexist in the same habitat, their interactions have a crucial effect on the configuration of the ecosystem. One such interaction is the trophic cooperation that arises when a compound excreted by one type of microorganism is used as a carbon source by another. For example, many microorganisms produce and excrete acetoin (3-hydroxy-2-butanone) when growing in environments that contain glucose or other fermentable carbon sources (Xiao & Xu, 2007). Not surprisingly, other microorganisms have acquired the ability to use this acetoin. In fact, acetoin is increasingly recognized as an important molecule in the trophic interactions between microorganisms. An important example is seen in the lungs of patients with cystic fibrosis when co-infected with *Staphylococcus aureus* and *Pseudomonas aeruginosa* or with *Streptococcus pneumoniae* and *P. aeruginosa*. The acetoin excreted by *S. aureus* or *S. pneumoniae* in the lung can be efficiently catabolized by *P. aeruginosa*. This benefits both pathogens: one gets rid of a compound that can become toxic, while the other profits from a new carbon source (Camus et al., 2020; Sabra et al., 2022). Similar examples can be found in the rhizosphere, where the acetoin and 2,3-butanediol produced by *Bacillus sp*. as waste products can be catabolized by other plant-associated microorganisms, including several pseudomonads (Huang et al., 1994; Priefert et al., 1991; Xiao & Xu, 2007).

A pathway for the assimilation of acetoin has been characterized in *P. putida* KT2440, a strain with great

metabolic versatility that can colonize soils, the rhizosphere and aquatic systems (Belda et al., 2016; Nelson et al., 2002; Udaondo et al., 2016; Wu et al., 2011). Its metabolism and physiology have been studied for decades which, together with its lack of virulence, has facilitated its use in many biotechnological applications (Nikel et al., 2014; Weimer et al., 2020). *Pseudomonas putida* assimilates acetoin using the acetoin dehydrogenase enzyme complex, which transforms acetoin into acetaldehyde and acetyl-CoA (Figure 1A; reviewed in the study by Spalding & Prigge, 2010). The genes encoding the different subunits of acetoin dehydrogenase are clustered and ordered as *acoX*, *acoA*, *acoB* and *acoC* (Figure 1B; Huang et al., 1994). The *acoX* gene codes for a protein of unknown function, while *acoABC* codes for the E1α, E1β and E2 subunits of the acetoin dehydrogenase complex. Downstream of *acoC* is a gene that produces an alcohol dehydrogenase that oxidizes 2,3-butanediol to acetoin (Liu et al., 2021).

Little is known about the regulation of acetoin catabolism in *Pseudomonas*. In *Alcaligenes eutrophus* H16, which contains a similar gene cluster for acetoin

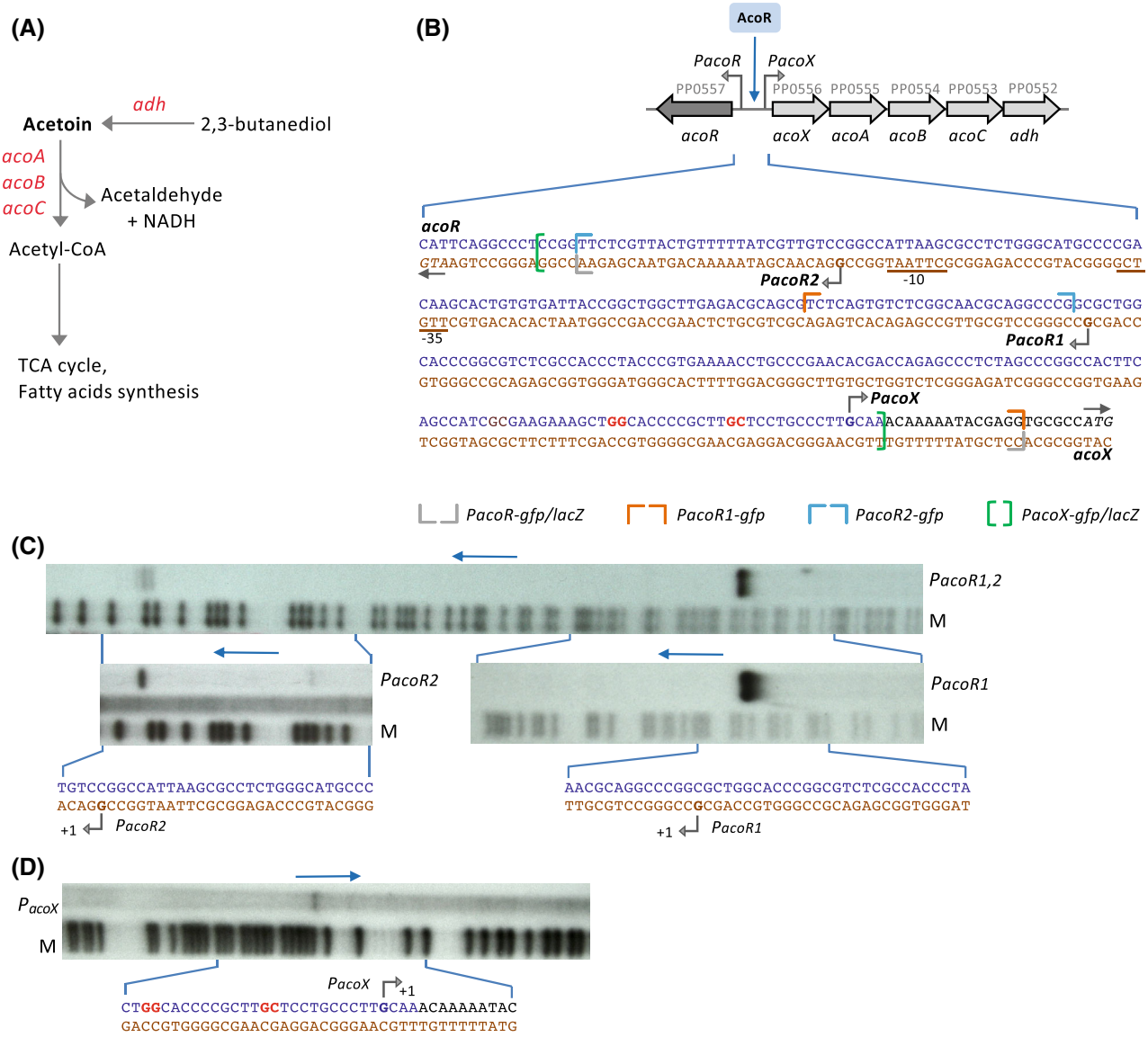

FIGURE 1 Identification of the promoters of the *P. putida* KT2440 acetoin assimilation genes. (A) Diagram of the enzymatic steps and genes (in red) involved in the assimilation of acetoin. (B) Organization of the acetoin degradation genes. The promoters responsible for the expression of the *acoXABC* genes (*PacoX*) and the *acoR* transcriptional regulator (*PacoR1* and *PacoR2*) are indicated by arrows and correspond to those deduced from Figure 1C,D. At *PacoX*, the GG and GC dinucleotides characteristic of RpoN-dependent promoters are highlighted in bold/red. Coloured brackets indicate the DNA regions used to construct the reporter fusions *PacoX-gfp* and *PacoX-lacZ* (green brackets), *PacoR1-gfp* (brown brackets), *PacoR2-gfp* (blue brackets), and *PacoR-gfp* and *PacoR-lacZ* (grey brackets; in the direction opposite to that used with *PacoX-gfp*). (C, D) Identification of the transcriptional start sites of the *acoR* and *acoX* genes by S1 nuclease protection assays; 'M' denotes a sequence ladder obtained by chemical sequencing of the end-labelled DNA fragment used in the assay.

assimilation, transcription of the *acoXABC* genes occurs from a promoter that requires the RpoN sigma factor and the AcoR transcriptional regulator (Krüger & Steinbüchel, 1992; Priefert et al., 1991). In *P. putida* LU6456, a gene similar to *A. eutrophus* H16 *acoR* is present upstream of the *acoXABC* genes and believed to activate their transcription (Pedroni et al., 2002). A similar gene organization is found in *P. putida* KT2440 (Figure 1B), although the orientation of *acoR* relative to *acoX* is the opposite of that seen in *A. eutrophus*. In *P. putida* PpG2, acetoin dehydrogenase is induced by acetoin or 2,3-butanediol, but no enzyme activity is detected if cells are cultured on glucose or acetate (Huang et al., 1994). In *P. aeruginosa* PAO1, the acetaldehyde released from acetoin oxidation was found to be a direct effector of the AcoR transcriptional activator (Liu et al., 2018).

Under the conditions encountered in soils and the rhizosphere, acetoin is unlikely to be the only carbon source present. Depending on the metabolic preferences of the microorganisms in the considered environment, some of these compounds will be catabolized simultaneously and others sequentially (Bajic & Sánchez, 2020). The combination of nutrients available, the biochemical conflicts that their assimilation may elicit, the nutrient preferences of the different microbes present, and the trophic interactions that can emerge between them, together influence the composition of the final microbial community (Estrela et al., 2021; Goldford et al., 2018; Johnson et al., 2012). Nutrient preferences differ among bacterial groups. Many prefer sugars such as glucose over other compounds, while some prefer particular amino acids or organic acids. The complementarity of such opposing strategies allows bacteria to specialize on a given carbon source, reducing competition with other microbes (Park et al., 2020). It is therefore important to understand how bacteria coordinate and regulate the assimilation of the different compounds present in their habitats (Moreno & Rojo, 2023).

In pseudomonads, the uptake and assimilation of nutrients are coordinated by different global regulatory systems that allow cells to adjust their metabolism to their situation (Rojo, 2010; Shingler, 2003). One of these relies on the combined action of the Hfq and Crc proteins, the CrcZ and CrcY small RNAs (sRNAs), and the CbrB regulator. When cells are confronted with different carbon sources at high enough concentrations, this regulatory system facilitates their ordered and sequential uptake and assimilation (García-Mauriño et al., 2013; La Rosa et al., 2016; Molina et al., 2019; Moreno et al., 2015; Sonnleitner et al., 2018; Valentini et al., 2014; Yuste & Rojo, 2001). Hfq is an important post-transcriptional regulator in many bacterial groups that generally works by assisting the annealing of regulatory sRNAs to their target mRNAs (reviewed in the studies by Updegrove et al., 2016; Vogel & Luisi, 2011; Wagner & Romby, 2015). Hfq also functions in this way in *Pseudomonas* (Ferrara et al., 2015; Sánchez-Hevia et al., 2018), but it can also recognize A-rich motifs such as AAnAAnAA in mRNAs, forming unstable complexes that become stabilized after incorporating the Crc protein. When these motifs are located at the translation initiation region of a mRNA, translation is inhibited (Malecka et al., 2021; Moreno et al., 2015; Sonnleitner et al., 2018). The combined action of Hfq and Crc modulates the expression of many genes related to carbon metabolism, helping to optimize growth (Molina et al., 2019; Moreno et al., 2009). The activity of the Crc and Hfq proteins is antagonized by the CrcZ and CrcY sRNAs, which contain several A-rich motifs that bind and sequester the Crc and Hfq proteins (Hernández-Arranz et al., 2016; Madhushani et al., 2015; Moreno et al., 2012, 2015; Sonnleitner et al., 2009; Sonnleitner & Bläsi, 2014). The levels of CrcZ and CrcY vary depending on the carbon sources present and/or on physiological conditions, being lower when cells use preferred carbon sources (catabolite repression is strong), and higher when non-preferred carbon sources have to be used (catabolite repression is weak) (Valentini et al., 2014 and references cited above). The transcription of *crcZ* and *crcY* is controlled by the CbrA/CbrB two-component regulatory system, which responds to still unclear nutritional cues (García-Mauriño et al., 2013; Valentini et al., 2014).

Other global regulatory systems are known that can impact the expression of certain genes involved in carbon assimilation. One of them is associated with the Cyo terminal oxidase, a component of the electron transport chain that plays an important role as a final electron acceptor and proton pump under highly aerobic conditions (Arai et al., 2014; Williams et al., 2007). The inactivation of Cyo leads to compensatory modifications in the composition of the electron transport chain (Morales et al., 2006) and to increased induction of the genes involved in the assimilation of carbon sources such as phenol (Petruschka et al., 2001) or *n*-alkanes (Dinamarca et al., 2002, 2003). The underlying mechanism is unknown but apparently links the activity of the electron transport chain to the expression of particular genes.

Another regulatory system, named PTS^{Ntr}, is formed by an array of three proteins ($PtsP/EI^{Ntr}$, PtsO/NPr and $PtsN/EIIA^{Ntr}$) that can be sequentially phosphorylated or dephosphorylated. Phosphoenolpyruvate is the initial donor of the phosphoryl group (Chavarría et al., 2012). The PtsN protein, most likely in its non-phosphorylated form, may directly or indirectly influence the activity of particular proteins, influencing the carbon flow between certain catabolic pathways and the tricarboxylic acid (TCA) cycle, helping in this way to adjust metabolic fluxes to cellular needs. In response to the presence of glucose, the PTS^{Ntr} system inhibits the induction of the *P. putida* genes for the assimilation of

toluene carried by the pWW0 plasmid (Aranda-Olmedo et al., 2005, 2006; Cases et al., 1999). This regulation relies on the level of 2-keto-3-deoxy-6-phosphogluconate, an intermediate of the Entner–Doudoroff pathway for glucose assimilation (del Castillo & Ramos, 2007; Velázquez et al., 2004).

The aim of the present work was to better understand the regulation of the *P. putida* KT2440 acetoin catabolism genes and to determine whether the simultaneous presence of alternative carbon sources can impair acetoin degradation. The results show that the induction of these genes by acetoin is down-modulated by nutritional cues that are sensed by several overlapping global regulatory systems.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture media

Cells were cultured in LB (10 g/L tryptone; 5 g/L yeast extract, 10 g/L NaCl) or in M9 minimal salts medium (Sambrook & Russell, 2001) supplemented with trace elements (Bauchop & Eldsen, 1960); the final composition of the supplemented medium was 42.4 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 25 μM $CaCO_3$, 1.2 μM $CoSO_4$, 1.3 μM $CuSO_4$, 42.7 μM $FeSO_4$, 0.4 μM H_3BO_3, 776 μM HCl, 333 μM MgO, 38.1 μM $MnSO_4$, and 6.25 μM $ZnSO_4$. The M9 minimal salts medium was amended with 30 mM succinate, 30 mM glucose, 30 mM citrate, 30 mM pyruvate, 30 mM arginine, or 15 mM acetoin, as indicated, as carbon source. When needed, antibiotics were added at the following concentrations: kanamycin 50 μg/ml, streptomycin 50 μg/ml, tetracycline 8 μg/ml, and ampicillin 100 μg/ml. Cell growth was followed by measuring turbidity at 600 nm. *Escherichia coli* strains were cultured at 37°C and *P. putida* strains at 30°C.

The *E. coli* strains used were DH5α (Woodcock et al., 1989), HB101 (pRK600) (Kessler et al., 1992), and CC118λpir (Herrero et al., 1990). The *P. putida* strains used have been previously described: KT2440 (Franklin et al., 1981), KT2440-*rpoN*::*km* (Köhler et al., 1989), KT2440-IHF3 (Marqués et al., 1998), KTVC (a *crc*::*aacC1* derivative of strain KT2440; Sánchez-Hevia et al., 2018), KT2440-ZY (which lacks the *crcZ* and *crcY* genes; La Rosa et al., 2015), KT2440B1 (a *cyoB*::*tet* derivative of strain KT2440; Sevilla et al., 2013), and KT2440/*ptsN* (a *ptsN*::*km* derivative of strain KT2440; Aranda-Olmedo et al., 2005).

Derivatives of strain KT2440 with inactivated *acoR* or *acoB* genes (strains KT2440/*acoR* and KT2440/*acoB*, respectively) were obtained by allele exchange as follows. A ~1000 bp DNA fragment containing *acoR* was PCR amplified with oligonucleotides mut-acoR-dir and mut-acoR-inv (Table S1) and cloned into plasmid pGEM-T Easy (Promega), generating plasmid pGEM-acoR. A tetracycline resistance determinant, obtained by digesting plasmid pUT-miniTn5Tc (Herrero et al., 1990) with SmaI, was purified and cloned into the NruI site of the *acoR* gene of pGEM-acoR. A NotI DNA fragment containing *acoR*::*tet* was excised from the resulting plasmid and cloned into the NotI site of the suicide plasmid pKNG101 (Kaniga et al., 1991), generating plasmid pKNGacoR-Tc. This plasmid was introduced into *P. putida* KT2440 by conjugation, as previously described (de Lorenzo & Timmis, 1994). Transconjugants were selected by growth on M9 minimal salts medium agar plates containing citrate as the sole carbon source and tetracycline. The presence of an inactivated *acoR-tet* allele, and the absence of the wild-type allele, was verified by PCR. A KT2440 derivative with an inactivated *acoB* allele was obtained following a similar procedure, using oligonucleotides acoB-dir and acoB-rev (Table S1), and interrupting the *acoB* gene at its NarI site (blunt ended with T4 DNA polymerase) with the same tetracycline resistance determinant.

Identification of the *acoR* and *acoX* transcription start sites by S1 nuclease protection assays

Total RNA was isolated from cells cultured at 30°C in aerated flasks containing LB medium supplemented with 5 mM acetoin. At late-exponential phase ($A_{600} = 0.8$), 20 ml of samples was collected, harvested by centrifugation, and frozen at −70°C. Total RNA was purified from cell pellets using the RNeasy RNA purification kit (QIAGEN). Purified RNA was treated with RNase-free DNase I (TURBO DNA-free, Ambion), as indicated by the manufacturer. The absence of DNA in the RNA preparations was tested by real-time PCR using primers for the *rpoN* gene as previously described (Morales et al., 2006).

S1 nuclease protection reactions were performed as previously described (Sevilla et al., 2017). Briefly, a DNA fragment containing the promoter region analysed was PCR-amplified with a suitable primer pair (indicated in Table S1) in which the reverse primer had been radioactively labelled at its 5′-end using [γ^{32}P]-ATP and T4 polynucleotide kinase. The end-labelled DNA fragment (50,000 cpm) was mixed with 25 μg of RNA, precipitated with ethanol, and the single-stranded DNA regions digested with 40 units of S1 nuclease as indicated by the provider (Thermo-Fischer Scientific). The undigested DNA was ethanol-precipitated and analysed in a denaturing urea-polyacrylamide gel, side by side with a DNA sequence ladder obtained by chemical sequencing of a 5′ end-labelled DNA fragment, as previously described (Maxam & Gilbert, 1980). In brief, the DNA was treated with formic acid, which reacts with

purines (adenine and guanosine), at a concentration that introduced, on average, one modification per DNA molecule. The modified DNA was cleaved at the position of the modified base by treatment with hot piperidine, rendering a series of labelled fragments that can be used as sequence ladder.

Construction of transcriptional fusions of the promoters for the *acoR* and *acoX* genes to the *lacZ* and *gfp* reporter genes

To obtain transcriptional fusions of the promoters *PacoX* and *PacoR* to the *lacZ* reporter gene, DNA fragments containing the corresponding promoter regions were PCR-amplified with the oligonucleotide pairs PacoX-BamHI/PacoX-Hind and PacoR-EcoRI/PacoR-Hind, respectively (sequences provided in Table S1), using chromosomal DNA obtained from *P. putida* KT2440 as a template. The DNA fragments obtained were digested with the endonucleases BamHI and HindIII (for *PacoX* promoter), or EcoRI and HindIII (for *PacoR* promoter) and cloned between the equivalent sites of plasmid pSEVA225 (Martínez-García et al., 2015) to generate a transcriptional fusion to the *lacZ* reporter gene. Plasmid pSEVA225 has the RK2 replication origin and a kanamycin resistance determinant. The reporter plasmids obtained were named p225-acoX and p225-acoR. The final fusions included DNA segments spanning positions −21 to −254 relative to the *acoX* translation initiation codon, or −14 to −277 relative to the *acoR* translation initiation codon. Similar transcriptional fusions to the *gfp* gene were also constructed. For this, the promoter regions were excised from plasmids p225-acoX and p225-acoR with the endonucleases indicated above and cloned

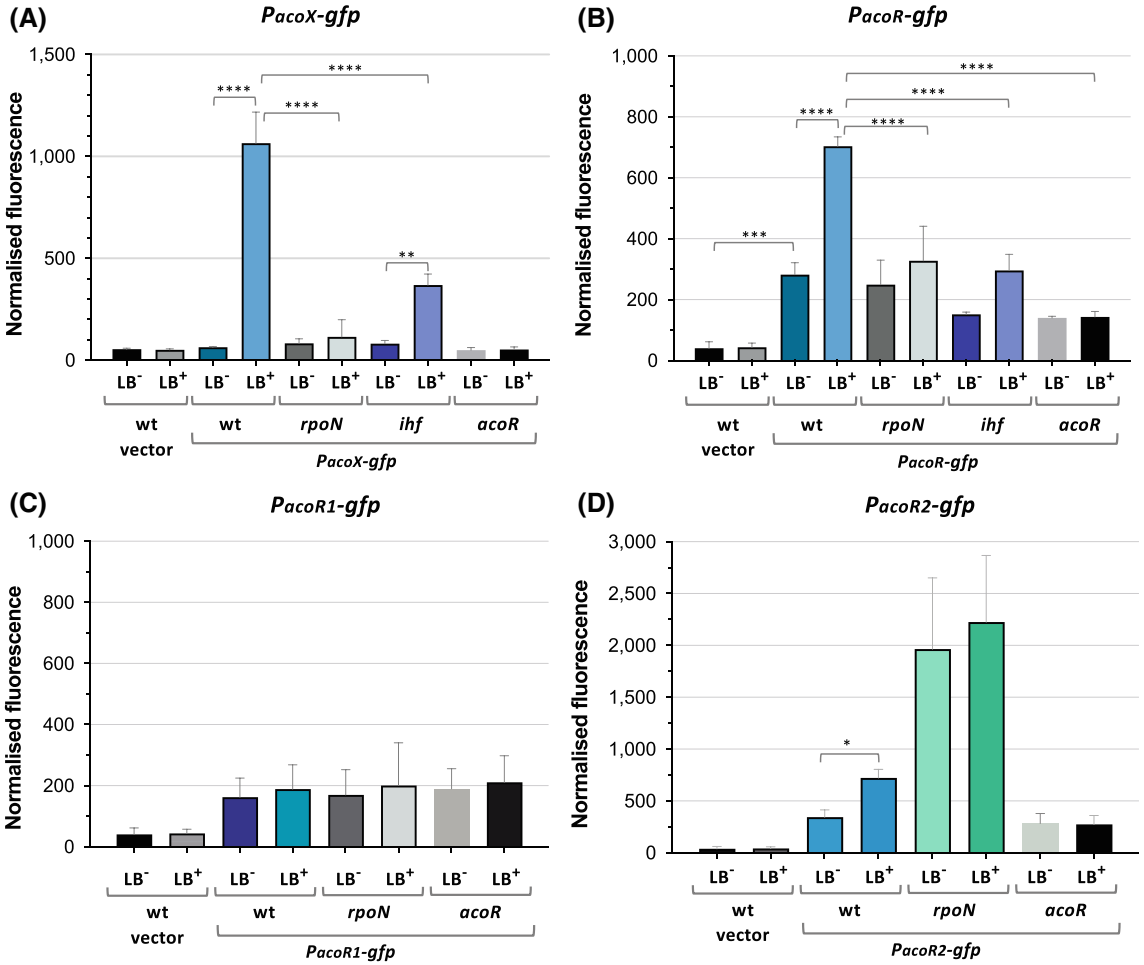

FIGURE 2 Influence of RpoN, IHF and AcoR on the activity of promoters *PacoX* and *PacoR*. The wild-type *P. putida* KT2440 strain, or its mutant derivatives with inactivated *rpoN*, *ihfA* or *acoR* alleles, containing the reporter fusions *PacoX-gfp* (A), *PacoR-gfp* (B; it includes promoters *PacoR1* and *PacoR2*), *PacoR1-gfp* (C), or *PacoR2-gfp* (D), were cultured in LB medium in the presence or absence of 5 mM acetoin, and GFP-derived fluorescence measured when cells reached the stationary phase (20 h culture in a 96-well plate at 30°C with agitation). The fluorescence detected was normalized for the turbidity of the culture. That obtained for strain KT2440 containing the empty plasmid vector used (contains the *gfp* gene, but no promoter) is also indicated, as a control. The mean and standard deviation of three independent assays are represented. Statistically significant changes are indicated (*$p < 0.05$; ***$p < 0.01$; ****$p < 0.0001$; one-way ANOVA). The DNA regions included in each reporter fusion are indicated in Figure 1B.

between the corresponding sites of plasmid vector pSEVA427 (Martínez-García et al., 2015) to generate transcriptional fusions to *gfp*. The plasmids obtained were named p427-acoX and p427-acoR and included an RK2 replication origin and a streptomycin resistance determinant.

Finally, transcriptional fusions to the *gfp* gene including either promoter *PacoR* -but lacking *PacoR2*-, or promoter *PacoR2*- but lacking *PacoR1*-, were constructed following a similar approach to that described above and using the oligonucleotides indicated in Table S1. The reporter plasmids obtained were named p427-acoR1 and p427-acoR2, respectively. The DNA region included in each fusion is presented in Figure 2A. Constructs were verified by DNA sequencing.

Construction of a translational fusion of the *acoX* gene to the *′lacZ* reporter gene

To generate a translational fusion of *acoX* to the *′lacZ* reporter gene, a DNA segment was PCR-amplified using *P. putida* KT2440 chromosomal DNA and the oligonucleotides PacoX-BamHI and acoX-trans-Hind (Table S1). After digestion with endonucleases BamHI and HindIII, the resulting fragment was cloned between the same sites of plasmid pSEVA225T (Martínez-García et al., 2015), generating a translational fusion that comprised positions −254 to +9 relative to the *acoX* translation initiation codon. This includes promoter *PacoX*, the translation initiation region, and the first three codons of *acoX*, fused in frame to the *′lacZ* coding sequence. It was named p225T-acoX. Constructs were verified by DNA sequencing.

Construction of translational fusions of *acoX* and *acoR* to the *′lacZ* reporter gene, in which the former genes are transcribed from the *Ptrc* promoter

To uncouple translational regulation from possible transcriptional effects, post-transcriptional fusions of *acoX* and *acoR* were made in which the *acoX′-′lacZ* or *acoR′-′lacZ* reporter constructions are transcribed from the heterologous promoter *Ptrc* after the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The *acoX′-′lacZ* translational fusion was PCR-amplified using plasmid p225T-acoX as a template, and the oligonucleotides acoX-dir-Eco-PT (which hybridizes upstream of *acoX′*) and lacZ-rev-Pst (which hybridizes downstream of *′lacZ*; Table S1). The DNA fragment generated was digested with EcoRI and PstI and cloned between the same sites of plasmid pSEVA424, generating plasmid p424-acoX, which contains a translational fusion including positions −35 to +9 relative to the *acoX* translation initiation codon, that is, it includes the first three *acoX* codons fused in frame to *′lacZ*.

A similar strategy was followed for *acoR*. As a first step, a DNA segment was PCR-amplified from the *P. putida* KT2440 chromosome using the oligonucleotides acoR-trans-Hind and PacoR-EcoRI (Table S1). After digesting this with the endonucleases EcoRI and HindIII, the resulting fragment was cloned between the same sites of plasmid pSEVA225T to generate a translational fusion comprising positions −277 to +9 relative to the *acoR* translation initiation codon, and which included the first three codons of *acoR* followed by the *′lacZ* coding sequence, in frame. This was named p225T-acoR. In a second step, directed towards substituting the promoter *PacoR1,2* by *Ptrc*, the *acoR′-′lacZ* translational fusion was PCR-amplified using plasmid p225T-acoR as a template, and the oligonucleotides acoR-dir-Eco-PT (which hybridizes upstream of *acoR′*) and lacZ-rev-Pst (which hybridizes downstream of *′lacZ*). The DNA fragment generated was digested with EcoRI and PstI and cloned between the same sites of plasmid pSEVA424, which bears the *Ptrc* promoter upstream of the EcoRI site. The resulting plasmid, named p424-acoR, contained a translational fusion that includes positions −42 to +9 relative to the *acoR* translation initiation codon. Constructs were verified by DNA sequencing.

β-galactosidase assay

An overnight culture of the strain containing the reporter plasmid of interest was diluted to a turbidity (A_{600}) of 0.05 in LB medium, or in M9 minimal salts medium containing the carbon source indicated in each case, plus the appropriate antibiotic to prevent plasmid loss. Cells were allowed to grow at 30°C with vigorous aeration; aliquots were taken at various time points and the β-galactosidase activity measured using *o*-nitrophenyl-β-D-galactoside as a substrate (Miller, 1972). All assays were performed in triplicate (at least).

Assays for green fluorescent protein

An overnight culture of the indicated bacterial strain was diluted in the appropriate fresh culture medium to a turbidity (A_{600}) of 0.05, and 150 μl dispensed (with triplicate technical replicates) onto a black, flat, clear-bottomed 96-well microtiter plate. Plates were incubated for 20 h at 30°C with agitation. The fluorescence (excitation 480 nm and emission 520 nm) and absorbance (600 nm) were measured simultaneously every 30 min. The fluorescence values recorded were normalized by the absorbance (number of cells). Assays were performed in triplicate, each with three technical replicates.

RESULTS

Characterization of the promoters of the acetoin assimilation genes

As a first step in examining the regulation of the *P. putida* KT2440 *aco* genes, the transcription start sites of the *acoX* and *acoR* genes were determined by S1 nuclease protection assays. The start site for *acoX* was located 25 nt upstream of its translation initiation codon (Figure 1B,D). The −24 and −12 regions upstream of this site showed the highly conserved GG-N_{10}-GC sequence motif characteristic of promoters recognized by the RpoN sigma factor (Barrios et al., 1999); this is in agreement with findings for the equivalent gene in *Alcaligenes eutrophus* H16 (Krüger & Steinbüchel, 1992; Priefert et al., 1991). This promoter was named *PacoX*. For *acoR*, two protected bands were detected, suggesting the presence of two transcription start sites. The first was 138 nt upstream of the *acoR* translation initiation codon; the corresponding putative promoter was named *PacoR1* (Figure 1B,C). The second start site was 40 nt upstream of the *acoR* translation initiation codon; the promoter was named *PacoR2* (Figure 1B,C). A motif resembling the consensus sequence for the sigma-70 factor was discernible for promoter *PacoR2* but not for *PacoR1* (Figure 1B).

The possible influence of the RpoN sigma factor on the activity of the promoters of *acoX* and *acoR* was analysed using transcriptional fusions to the *gfp* reporter gene. These were constructed by cloning the DNA regions indicated in Figure 1B into the plasmid vector pSEVA227. The resulting reporter plasmids were introduced into *P. putida* KT2440 (wild type) and its RpoN-null mutant derivative KT2440-*rpoN*::*km*. Fluorescence was recorded in cells cultured in microtiter plates containing the complete medium LB, with or without 5 mM acetoin. Preliminary assays showed that this acetoin concentration rendered a strong induction of the *PacoX-gfp* reporter fusion, while higher concentrations showed some toxicity, reducing growth rate. In addition, acetoin concentrations in glucose-grown *P. aeruginosa*, *S. aureus* or *Bacillus subtilis* culture supernatants can reach the millimolar range (Ali et al., 2001; Camus et al., 2020). For the wild-type strain with the *PacoX-gfp* fusion, fluorescence was low during the exponential phase of growth but increased by a factor of over 16 ($p < 0.0001$) when cells reached the stationary phase and acetoin was present in the medium, an increase that was not seen if acetoin was omitted; results are presented in Figure 2A for cells in the early stationary phase of growth (A_{600} of 1.2). Induction of the *PacoX-gfp* fusion by acetoin did not occur when the same fusion was introduced into the RpoN-null strain (Figure 2A). Further, the fluorescence detected for the RpoN strain was very similar to that seen for the wild-type strain containing the control plasmid lacking the *PacoX* promoter. Many RpoN-dependent promoters require the IHF protein to facilitate proper contact between the transcriptional activator (AcoR in this case) and the RpoN-RNA polymerase (Wigneshweraraj et al., 2008). Introduction of the *PacoX-gfp* reporter fusion into strain KT2440-IHF3, a KT2440 derivative that lacks IHF, reduced *PacoX* induction by acetoin to a third, which supports the idea that IHF helps AcoR to activate transcription. Therefore, *PacoX* behaves as an RpoN-dependent promoter. To confirm that AcoR is the transcriptional activator responsible for acetoin-dependent induction of promoter *PacoX*, the *acoR* gene of strain KT2440 was inactivated and the *PacoX-gfp* reporter fusion introduced into the resulting strain, which was named KT2440/*acoR*. Acetoin was unable to induce promoter *PacoX* in the AcoR-null background (Figure 2A). In addition, the *acoR* mutant strain was unable to grow using acetoin as the sole carbon source.

The importance of promoters *PacoR1 and PacoR2* in *acoR* expression was studied with the help of three transcriptional fusions to the *gfp* reporter gene, cloned into the plasmid vector pSEVA227. The first, named *PacoR-gfp*, contained a DNA fragment including both the *PacoR1* and *PacoR2* promoters. The other two, named *PacoR1-gfp* and *PacoR2-gfp*, contained smaller DNA fragments covering just one of the two promoters, as detailed in Figure 1B. In the absence of acetoin, the fluorescence detected for the wild-type cells containing the *PacoR-gfp* fusion was seven times that of cells containing the empty plasmid vector (Figure 2B). In the presence of acetoin, the activity of the *PacoR-gfp* fusion increased by a factor of ∼2.5. In the RpoN-null strain, and in the absence of acetoin, the activity recorded was similar to that observed in the wild-type strain, but it did not increase when acetoin was added to the culture (Figure 2B). The absence of IHF had an effect similar to the lack of RpoN. Intriguingly, the activity of the *PacoR-gfp* fusion was reduced to some 50% in the strain containing the inactivated *acoR* allele, irrespective of the presence of acetoin. In summary, the combined action of the promoters *PacoR1* and *PacoR2* has a significant basal activity that is independent of RpoN and increases in the presence of acetoin via the effect of AcoR.

The behaviour of the *PacoR1-gfp* reporter fusion suggests that promoter *PacoR1* activity is independent of the presence of acetoin, RpoN or AcoR (Figure 2C). In fact, this promoter does not show the consensus sites for the RpoN sigma factor. In the case of promoter *PacoR2*, the transcriptional output was similar to that of *PacoR1* in the absence of acetoin but increased by a factor of 2 if acetoin was present (Figure 2D). This increase was not seen in the AcoR-null strain. This

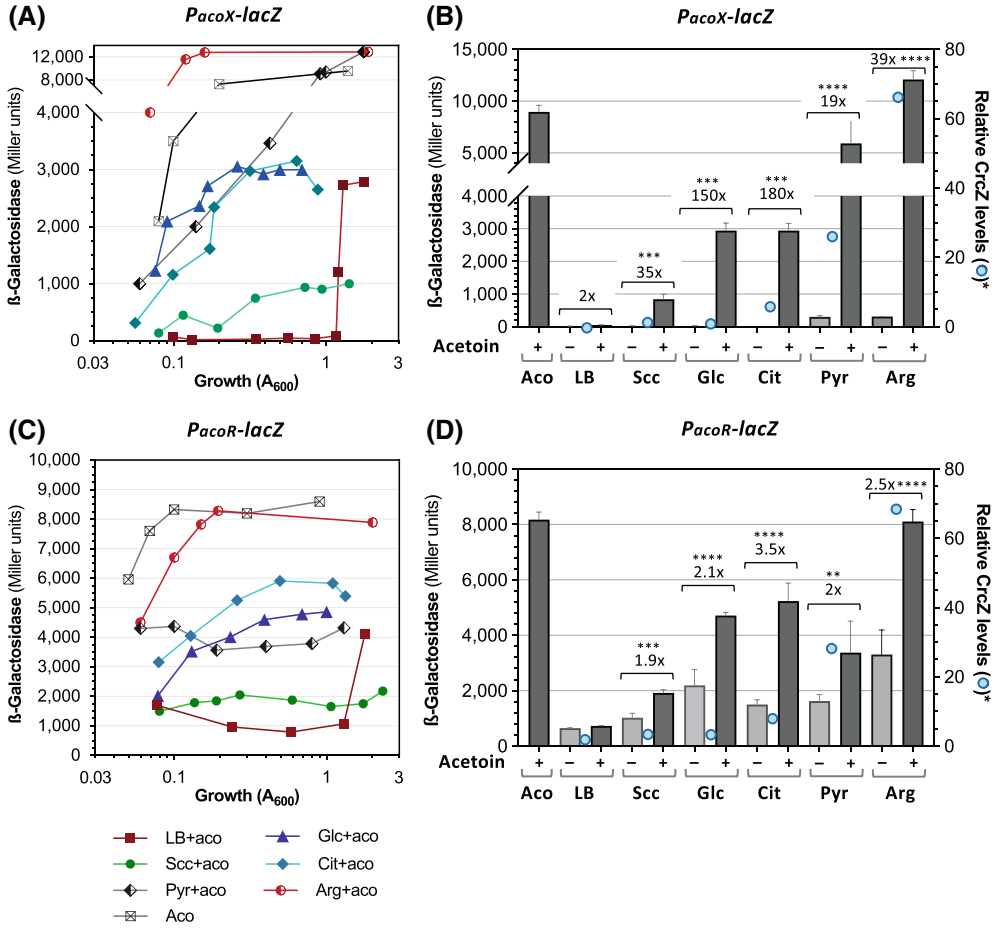

FIGURE 3 Induction of promoters *PacoX* and *PacoR* by acetoin in cells using different compounds as a carbon source. *Pseudomonas putida* KT2440 harbouring either a *PacoX-lacZ* (A, B) or a *PacoR-lacZ* (C, D) reporter transcriptional fusion, was cultured in flasks containing either LB medium, or M9 minimal salts medium with either acetoin (Aco), succinate (Scc), glucose (Glc), citrate (Cit), pyruvate (Pyr) or arginine (Arg) as the carbon source, in the later five cases in the presence (+) or absence (−) of acetoin. β-galactosidase activity was measured over time. Panels (A) and (C) represent the increase of β-galactosidase activity as cells grew in the presence of acetoin, or acetoin plus the indicated carbon source. Panels (B) and (D) show the β-galactosidase activity (mean and standard deviation of three independent assays) after cells reached a turbidity of 0.4–0.8 (mid-exponential phase), when cultured in the presence or absence of acetoin and the indicated carbon source. Blue dots indicate the CrcZ levels reported earlier (Valentini et al., 2014) in each of these growth conditions, expressed as a percentage of those recorded for cells using oxaloacetate as a carbon source. Significant changes are indicated (**p < 0.01; ***p < 0.001; ****p < 0.0001; one-way ANOVA).

suggests that AcoR activates *PacoR2*, but this promoter lacks the conserved consensus sites for the RpoN sigma factor, and its activity increased in an RpoN-null background, showing that AcoR cannot activate *PacoR2* in the way it activates *PacoX*. Promoter *PacoX* is not present in the *PacoR2-lacZ* fusion used to monitor the activity of *PacoR2* (see Figure 1B); the increase in the activity of *PacoR2* in the presence of acetoin cannot, therefore, be explained by some indirect interference of *PacoX* on *PacoR2*. The binding site for AcoR is not known, although it ought to lie somewhere between *PacoR2* and *PacoX*. If AcoR is able to bind to the upstream region of the *PacoR2-lacZ* fusion, it might facilitate *PacoR2* transcription in an RpoN-independent manner. Additional experimentation is required to see whether this is the case.

Influence of the carbon source on the expression of the acetoin degradation genes

Since acetoin coexists with other potential carbon sources in soils and the rhizosphere, tests were performed to see whether the metabolic configuration imposed by the presence of different carbon sources influences the expression of the acetoin degradation genes. For this, transcriptional fusions of the promoters *PacoX* and *PacoR* (including both *PacoR1* and *PacoR2*) to the *lacZ* reporter gene were used; these were identical to the transcriptional fusions used in Figure 2, except that the *gfp* reporter gene was substituted by *lacZ*. Note that, in these fusions, the translation initiation region is that of the *lacZ* reporter

gene but not that of *acoX* or *acoR*. They were, therefore, designed to detect transcriptional effects. However, since the activation of *PacoX* requires AcoR, which is provided by the chromosomal copy of this gene, any post-transcriptional effect on *acoR* expression might indirectly affect the activity of the *PacoX-lacZ* transcriptional fusion.

The growth medium had a strong influence on the induction profile of promoter *PacoX*. Figure 3 shows the results obtained. Panel A indicates the β-galactosidase activity over the growth curve in the presence of acetoin, or acetoin and the indicated carbon source, while panel B shows the activity detected in each medium at mid-exponential phase in the presence and absence of acetoin. When cells grew exponentially in a minimal-salts medium containing acetoin as the sole carbon source, the activity recorded for the *PacoX-lacZ* fusion was high (Figure 3A, grey rectangles, and lane 'Aco' in Figure 3B). If arginine, pyruvate, citrate or glucose were provided as a carbon source in addition to acetoin, *PacoX* induction was also strong. However, induction by acetoin was much weaker when succinate was present. When cells were cultured in LB medium, the addition of acetoin induced no transcription until the cells reached the stationary phase of growth (turbidity values of ∼1.2–1.4), at which time β-galactosidase activity increased strongly (Figure 3A). This suggests that the induction of promoter *PacoX* by acetoin is strongly inhibited during exponential growth in LB medium, an inhibition that rapidly fades away upon entry into the stationary phase.

The lesser induction of promoter *PacoX* during exponential growth in LB medium, or in minimal salts medium containing succinate, might be the consequence of a reduced expression of *acoR* under these growth conditions, leading to AcoR concentrations that cannot achieve the full induction of the *PacoX* promoter. To examine this possibility, the activity of a *PacoR-lacZ* transcriptional fusion was examined in cells cultured under the same conditions indicated above for *PacoX*. The results (see Figure 3C,D) suggest that *PacoR* activity varies depending on the growth medium and on the absence or presence of acetoin. Activity was lower in LB than in the other growth media used, but was still quite significant, and increased when cells entered the stationary phase of growth (Figure 3C). The presence of acetoin increased *PacoR* activity by a factor of 2–3, except when the growth medium was LB (Figure 3D). The overall picture suggests that the differences in *acoR* transcription observed with each medium only partially explain *PacoX* behaviour; other regulatory mechanism(s) would seem to be participating as well. For example, *acoR* expression might be modulated post-transcriptionally, which would impact the amount of AcoR available for *PacoX* activation. In fact, *PacoX* activity directly correlated with the transcription level of the CrcZ sRNA reported earlier for *P. putida* KT2440 cultured in the same growth media and carbon sources used here (Valentini et al., 2014) (represented in Figure 3B as blue dots; levels on the right Y axis). A similar, albeit weaker correlation was observed with respect to *PacoR* activity (Figure 3D). The levels of CrcZ, together with those of CrcY, control the repressing activity of the Crc/Hfq proteins, which are global regulators of metabolism in pseudomonads. The carbon source used strongly influences the amounts of CrcZ and CrcY present in the cell (Moreno et al., 2012; Valentini et al., 2014), which in turn determines the strength of the repression imposed by Hfq and Crc on the target genes. Repression is frequently directed towards the transcriptional activator of the genes involved in the transport or assimilation of the non-preferred carbon sources, although these genes might be direct targets as well (Hernández-Arranz et al., 2013). The translation initiation regions of *acoR* and *acoX* mRNAs include A-rich regions that could be potential targets for Crc/Hfq (Figure 4A). The possible influence of Crc/Hfq on the expression of the acetoin degradation genes was therefore investigated.

Influence of the Crc protein on *acoR* and *acoX* translation

A translational fusion was constructed that comprised *PacoX*, the 5′ region of *acoX* mRNA (including the native translation initiation sequences), and the start of the *acoX* open reading frame (first three codons), all fused in frame to the *′lacZ* reporter gene. The fusion, hereafter referred to as *PacoX-acoX′-′lacZ*, was introduced into a broad-host-range plasmid and transferred into *P. putida* KT2440 (wild type) and its Crc-null derivative KTVC. Cells were cultured either in LB medium with acetoin, or in minimal salts medium with acetoin, or a mixture of acetoin and either succinate, glucose, citrate, pyruvate or arginine. At mid exponential phase, samples were withdrawn and β-galactosidase activity measured. For cells cultured in minimal salts with acetoin, or with acetoin plus pyruvate or arginine, the activity observed was high, with values similar to those recorded for the wild type and Crc-null strains (Figure 4B). However, when cultured in LB with acetoin, or in minimal salts medium with acetoin plus succinate, glucose or citrate, the activity recorded for the Crc-null strain was higher than that noted for the wild-type strain. The effect was not large, but it was reproducible (Figure 4B). Since a lack of Crc did not fully relieve repression, the expression of the *aco* genes might also be controlled by other regulatory elements. A repressing effect of Crc/Hfq on the translation of the chromosomal copy of *acoR*, responsible for the activation of the *PacoX-acoX′-′lacZ* fusion, might also reduce the final output.

To examine the influence of the Crc/Hfq system on the expression of the *aco* genes, but avoiding any interference from transcriptional effects or from the chromosomal copy of *acoR*, a new reporter fusion was made in which the *acoX′-′lacZ* translational fusion was transcribed from the non-native *Ptrc* promoter, rather than from *PacoX*. Transcription from *Ptrc* can be induced upon the addition of IPTG. A similar fusion was made for *acoR*, which included the mRNA generated from *PacoR2* (but containing *Ptrc* rather than *PacoR2*) and the sequences up to the third codon of *acoR*, fused in frame to *′lacZ*. These fusions, named *Ptrc-acoX′-′lacZ* and *Ptrc-acoR′-′lacZ*, respectively, were cloned into a broad-host range plasmid and introduced into *P. putida* KT2440 and its Crc-null derivative KTVC. In cells cultured in LB containing IPTG, the activity of both reporter fusions in the Crc-null strain was about twice that recorded for the wild type (Figure 4C). This finding was reproducible and similar to that observed when the fusions were transcribed from the native promoters

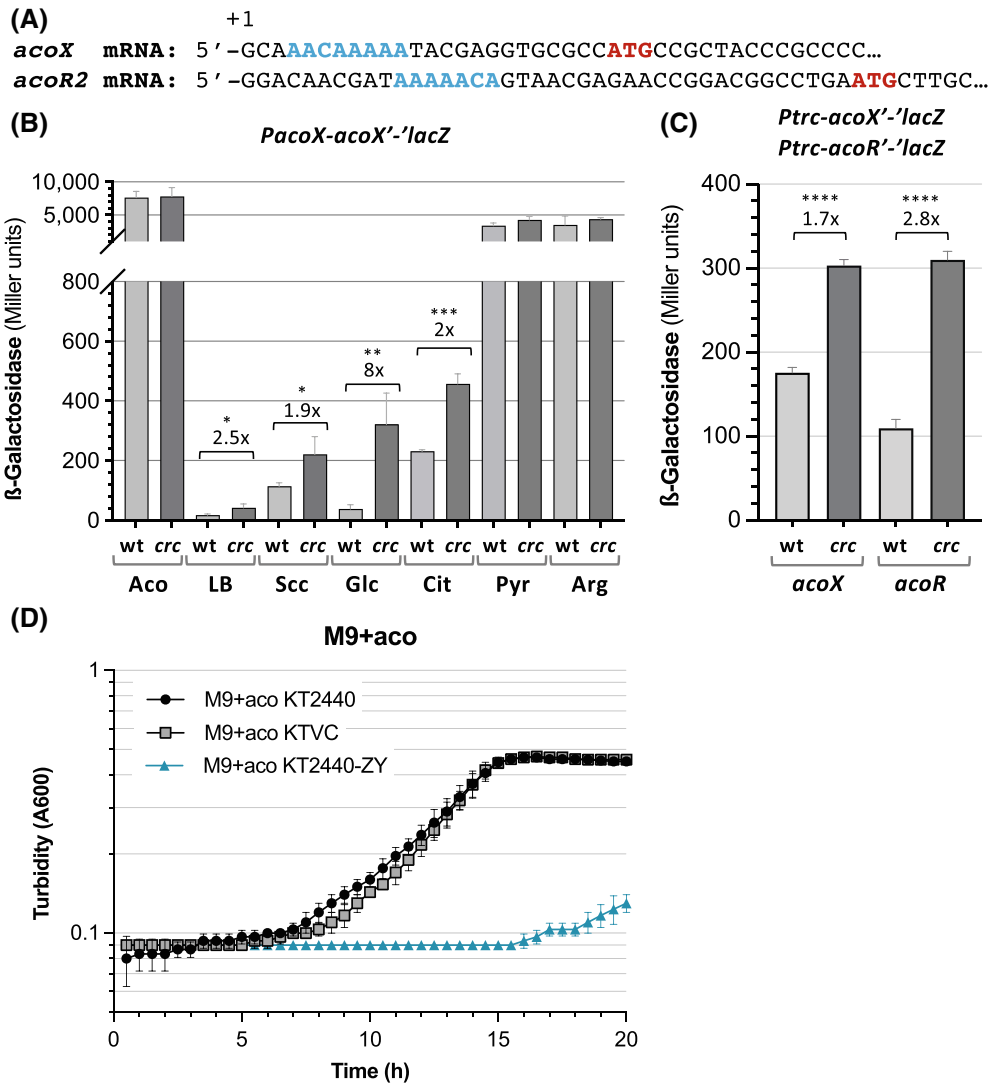

FIGURE 4 Influence of the Crc regulatory protein on the expression of the *acoX* and *acoR* genes. (A) 5′-end of the *acoX* and *acoR2* mRNAs; the ATG translation initiation codon is highlighted in red, while the A-rich sequence showing similarity to Hfq/Crc binding sites is highlighted in blue. (B) A plasmid containing the *PacoX-acoX′-′lacZ* reporter fusion was introduced into strains KT2440 (wt) or its Crc-null derivative KTVC (*crc*). Cells were cultured in LB medium containing acetoin, or in minimal salts medium containing acetoin and the indicated carbon source. The β-galactosidase activity observed at mid-exponential phase (turbidity 0.4–0.6) in cultures containing acetoin is indicated; that for cultures lacking acetoin was very low and is not shown. (C) Plasmids containing the reporter fusions *Ptrc-acoX′-′lacZ* or *Ptrc-acoR′-′lacZ* were introduced into strains KT2440 (wt) or its Crc-null derivative KTVC (*crc*). Cells were cultured in LB medium containing IPTG to induce promoter *Ptrc*. At mid-exponential phase (turbidity ~0.6), samples were taken and the β-galactosidase activity measured. The graphs indicate the mean and standard deviation for three independent assays. In panels (B) and (C), the ratio of the values obtained for the Crc-null strain relative to the wild type is indicated, together with the statistical significance of the differences observed (one-way ANOVA; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). (D) Growth of *P. putida* strains KT2440 (wild type), KTVC (Crc-null) and KT2440-ZY (CrcZ/CrcY-null) in a microtitre plate containing M9 minimal salts medium with acetoin as the sole carbon source

(compare Figure 4C with Figure 4B). A similar result was observed when cells were cultured in M9 minimal salts medium with glucose as the carbon source, and IPTG (not shown). It would therefore appear that the A-rich regions present in the translation initiation sites of *acoX* and *acoR* mRNAs are functional Hfq/Crc target sites that help control the translation of these mRNAs.

In further analysis of the repressing effect of Crc, the ability of the mutant strain KT2440-ZY to grow in acetoin was tested. This strain derives from KT2440 via the inactivation of the genes specifying the CrcZ and CrcY sRNAs, leading to high (unregulated) availability of the Crc protein, and to the over-repression of the Crc-regulated genes (Moreno et al., 2012). This mutant grows well in LB medium, under which conditions catabolite repression is strong, but shows impaired growth under conditions in which catabolite repression should be low level or absent such as in minimal salts medium with glucose, citrate or benzoate (Moreno et al., 2012). The unregulated action of Crc in this mutant strain inhibits the genes involved in the transport and assimilation of these compounds. As shown in Figure 4D, strain KT2440-ZY was unable to grow if acetoin was the sole carbon source, while the wild-type strain, or its Crc-null mutant, could do so.

Effect of the Cyo terminal oxidase on expression of the *aco* genes

The plasmid containing the *PacoX-lacZ* transcriptional fusion was introduced into strain KT2440B1, a derivative of KT2440 with an inactivated *cyoB* gene and therefore lacking the Cyo terminal oxidase. This is known to affect the expression of several genes via an as-yet unknown mechanism (Dinamarca et al., 2002, 2003; Morales et al., 2006; Petruschka et al., 2001). Strains KT2440 and KT2440B1 containing this reporter fusion were cultured in aerated flasks containing either LB medium or M9 minimal salts medium amended with succinate, glucose, citrate or pyruvate as a carbon source and in the absence or presence of acetoin. In cells growing exponentially, inactivation of the *cyoB* gene had no effect on *PacoX* activity when glucose, citrate or pyruvate were present, or when cells were cultured in LB medium (Figure 5). However, when succinate was the carbon source, *PacoX* activity in the presence of acetoin was greater in the *cyoB* mutant than in the wild-type strain by a factor of 2.6 ($p < 0.0001$).

Influence of the PTS^{Ntr} regulatory system on the expression of the *aco* genes

The effect of the PTS^{Ntr} regulatory system was analysed with the help of strain KT2440/*ptsN* (Aranda-Olmedo et al., 2005), in which the *ptsN* gene encoding one of the components of the PTS^{Ntr} system is inactivated by a kanamycin-resistance determinant. Due to antibiotic-resistance marker compatibility, the transcriptional reporter plasmid for *PacoX* promoter based on the GFP protein (plasmid p427-acoX), rather than that based on the LacZ protein, was introduced into this strain. The absence of the PtsN protein had little influence on *PacoX* activation by acetoin when cells were grown in M9 minimal salts medium supplemented with succinate, glucose, pyruvate, arginine or acetoin, and the small effect observed in the case of LB medium lacked statistical significance (Figure 6). However, when citrate was the carbon source used, the induction of promoter *PacoX* by acetoin doubled in the PtsN-null background (Figure 6).

Acetoin improves the growth rate when co-metabolized with pyruvate

The addition of 5 mM acetoin to a culture of strain KT2440 in LB, or in M9 minimal salts medium with glucose, citrate or succinate as the carbon source, barely affected growth. However, when pyruvate was the carbon source, the simultaneous presence of acetoin led

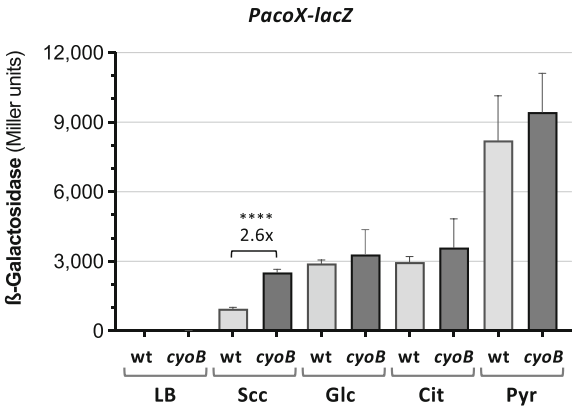

FIGURE 5 Influence of the Cyo terminal oxidase on the activity of promoter *PacoX*. Strains KT2440 (wt) and KT2440B1 (*cyoB*) containing plasmid p225-acoX, which includes a *PacoX-lacZ* transcriptional reporter fusion, were cultured in aerated flasks with either LB medium or M9 minimal salts medium amended with succinate, glucose, citrate, or pyruvate as the carbon source, and in the presence or absence of acetoin. At different times, samples were taken and the β-galactosidase activity determined. The graph shows the values observed at mid-exponential phase (turbidity 0.4–0.6) in cultures containing acetoin; those for cultures lacking acetoin were very low and are not shown. Values for LB-cultured cells were also very low and not visible at the scale used. For cells cultured in M9 + Scc and acetoin, the ratio of the values obtained for the *cyoB* mutant strain relative to the wild type is indicated, together with the statistical significance of the difference observed (one-way ANOVA; ****$p < 0.0001$). In all other comparisons, the differences were not significant. Values are the mean of three independent assays and the standard deviation is indicated.

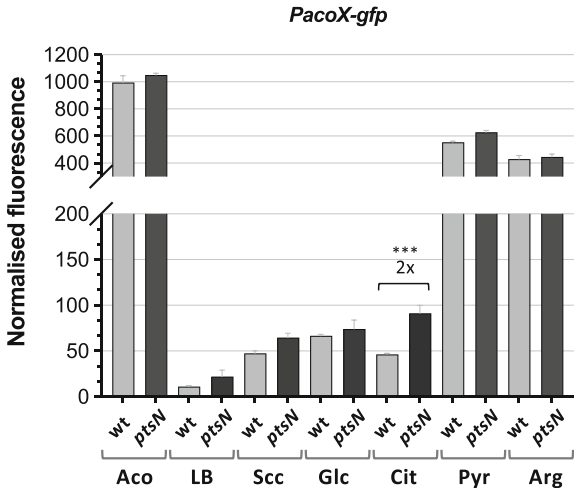

FIGURE 6 Influence of the PTS^{Ntr} system on the activity of promoter *PacoX*. Strains KT2440 (wt) and KT2440/*ptsN* (*ptsN*) containing plasmid p427-acoX, which includes a *PacoX-gfp* transcriptional reporter fusion, were cultured in 96-well microtiter plates containing either LB medium, or M9 minimal salts medium amended with succinate, glucose, citrate, pyruvate or arginine as the carbon source, and in the presence or absence of acetoin. The plates were introduced into a microtiter-plate reader and incubated for 48 h at 30°C with shaking, recording fluorescence and turbidity every 30 min. The graph shows the fluorescence values (normalized for turbidity) observed at mid-exponential phase (turbidity 0.5–0.6) in cultures containing acetoin; those of cultures lacking acetoin were very low and are not shown. Values are the mean of three independent assays; the standard deviation is indicated. For cells cultured in M9 + Cit + acetoin, the ratio of the value obtained for the *ptsN* mutant strain relative to the wild type is indicated, together with the statistical significance of the difference observed (one-way ANOVA; $p < 0.001$). In all other comparisons, the differences were not significant.

to a strong increase in the growth rate. Figure 7A–C shows the results obtained with cells growing in 96-well microtiter plates, in which the growth rate is slower than in aerated flasks but comparisons are easier and less open to misinterpretation. In fact, pyruvate alone was a poor carbon source (poorer than any of the others tested), but the simultaneous presence of acetoin and pyruvate led the growth rate to double, reaching values close to those recorded with glucose (Figure 7A). This growth advantage disappeared when the *acoB* gene was inactivated, a mutation that impairs acetoin assimilation (strain KT2440/*acoB*, Figure 7F).

Arginine was also a poor carbon source, although better than pyruvate. The simultaneous presence of acetoin improved growth by reducing the lag time (Figure 7B). Altogether, these observations suggest that acetoin can be co-metabolized with pyruvate and arginine, which confers a growth advantage.

DISCUSSION

The *P. putida* genes required for the degradation of acetoin, including the AcoR transcriptional regulator required for the expression of these genes, and a putative RpoN recognition site upstream of *acoX*, were reported earlier (Huang et al., 1994; Pedroni et al., 2002). In the present work, the transcription start site of *acoX* was determined, and the activity of the promoter *PacoX* was shown to require the RpoN sigma factor. In addition, optimal promoter activity was seen to require the IHF protein, which helps many RpoN-dependent activators make contact with RpoN-RNAP and form a nucleoprotein complex with the architecture needed to trigger transcription. Two transcription start sites were found for *acoR*, separated by 98 bp. Promoter *PacoR1* showed a basal activity that was independent of RpoN and AcoR and of the presence or absence of acetoin. Promoter *PacoR2* showed a similar basal activity that doubled in the presence of acetoin for reasons that remain unclear.

Although the expression of the acetoin degradation genes was strongly induced by acetoin, induction was reduced by the simultaneous presence of other carbon sources, indicating that acetoin is a non-preferred substrate and that its assimilation is controlled by global regulatory systems that help coordinate metabolism. Inhibition was strongest in cells growing exponentially in LB medium, in which acetoin was almost completely unable to induce transcription from promoter *PacoX* until the cells reached the stationary phase of growth, at which time promoter activity increased steadily. This behaviour has been observed for other *P. putida* RpoN-dependent promoters, such as the XylR-controlled *Pu* promoter (Cases & de Lorenzo, 2000) and the DmpR-controlled *Po* promoter (Sze et al., 1996), which drive the expression of genes involved in toluene and phenol degradation, respectively. This low promoter output during exponential growth in LB medium, and the sudden increase at the onset of the stationary phase, is known as 'exponential silencing' (Cases et al., 1996). Several factors were found to be involved, which did not affect the *Pu* and *Po* promoters to the same extent (Sze et al., 2002). One was the availability of the IHF protein, required in both cases for optimal promoter output. The amount of IHF during exponential growth in LB was found to be too low for any efficient activation of the XylR/*Pu* and DmpR/*Po* regulator/promoter pairs, but it increased significantly when cells entered the stationary phase, boosting promoter output (Valls et al., 2002). In the present work, IHF was found necessary for the efficient activation of promoter *PacoX* (Figure 2A); it might, therefore, play a role in the exponential silencing observed in LB medium.

A second factor has been traced to the nutritional alarmone ppGpp, which impacts the amount of free RNA polymerase core available to bind the RpoN sigma factor (Carmona et al., 2000; Shingler, 2003; Sze et al., 2002; Sze & Shingler, 1999). Overproducing RpoN partially relieved exponential silencing at the XylR/*Pu* promoter, showing that the amount of the

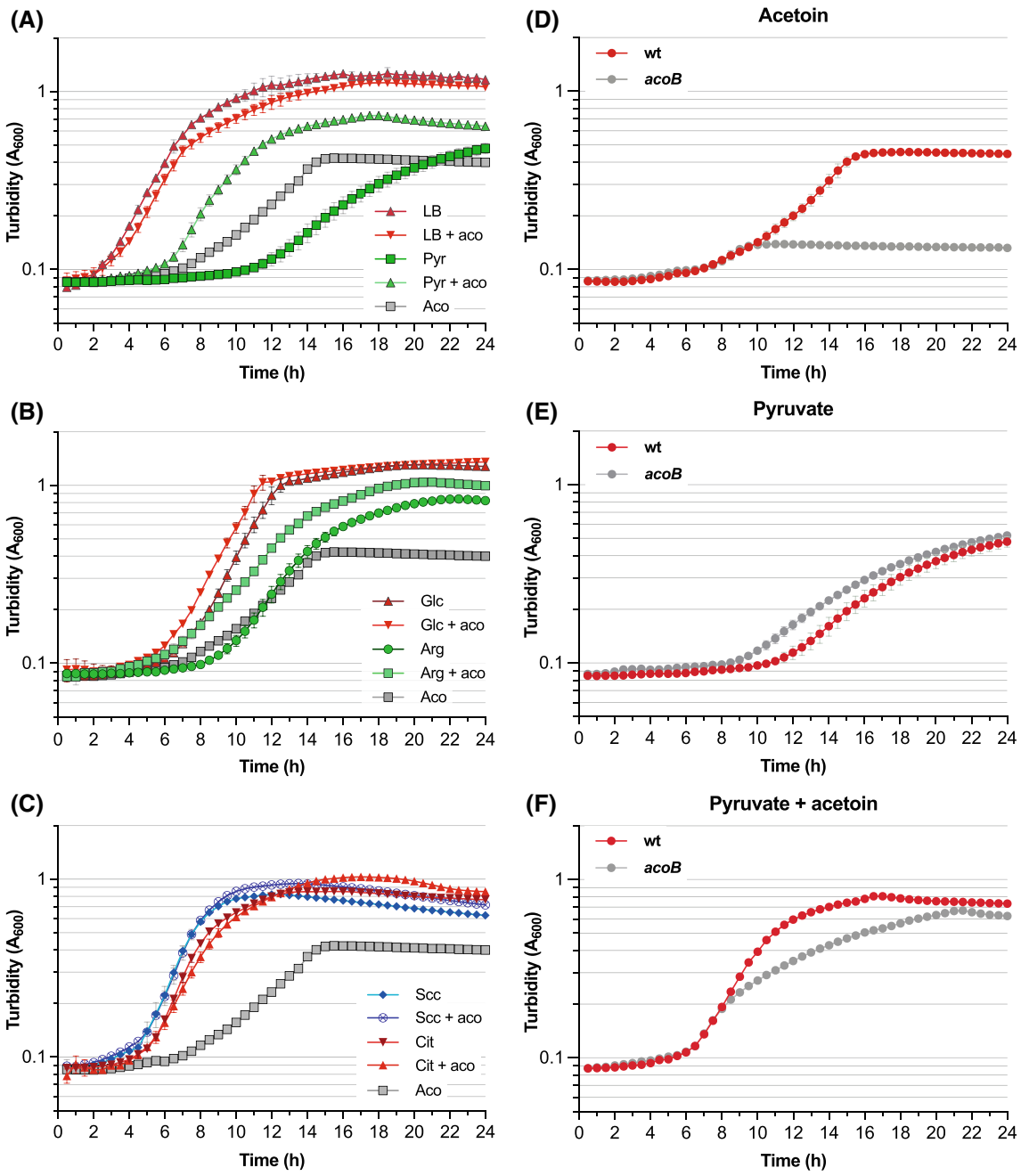

FIGURE 7 Influence of acetoin on growth in the presence of other carbon sources. Strains KT2440 or KT2440/*acoB* were inoculated into a clear, flat-bottom 96-well microtitre plate containing either LB medium or M9 minimal salts medium supplemented with succinate (Scc), glucose (Glc), citrate (Cit), pyruvate (Pyr), arginine (Arg), with or without acetoin (Aco). These plates were introduced into a microtiter-plate reader and incubated for 48 h at 30°C with shaking, recording turbidity (A_{600}) every 30 min. The graphs show the increase in turbidity detected as a function of time; values correspond to the mean (±standard deviation) of three cultures. Panels (A—C) show the growth of the wild-type strain KT2440 with the different carbon sources tested, while panels (D–F) compare the growth of strains KT2440 and KT2440/*acoB* with either acetoin, pyruvate, or a mixture of both.

RpoN-bound form of RNA polymerase is limiting during exponential growth in LB medium (Cases et al., 1996). The possible role of ppGpp in promoter *PacoX* activity was not specifically analysed here, but the availability of RpoN-bound RNA polymerase likely impacts promoter *PacoX* output as well.

A third element that limits the activity of XylR/*Pu* and DmpR/*Po* in LB medium is the Crc/Hfq regulatory system (Aranda-Olmedo et al., 2005; Madhushani et al., 2015; Moreno et al., 2010). Translation of the *xylR* and *dmpR* mRNAs is inhibited by the Crc/Hfq proteins, which limits the availability of the XylR and DmpR

activators, thereby impairing stimulation of their target promoters (Madhushani et al., 2015; Moreno et al., 2010). The present results show that the Crc/Hfq proteins inhibit the translation of *acoR* mRNA in cells growing exponentially in LB (Figure 4C) and therefore contribute to the regulation of expression of the acetoin degradation genes. Crc/Hfq also inhibited the translation of *acoX* mRNA (Figure 4C). A-rich regions similar to Crc/Hfq targets are also present at the translation initiation sites of the *acoA*, *acoB* and *adh* genes, although their functionality was not specifically addressed. The presence of Crc/Hfq targets on more than one gene involved in a catabolic pathway for a non-preferred compound has been previously noted (Hernández-Arranz et al., 2013; Moreno et al., 2010; Wirebrand et al., 2018).

In the defined growth media used, the induction of the promoter *PacoX* by acetoin was much more effective than in LB medium, but the induction ratio decreased in the presence of some alternative carbon sources. The inhibitory effect of succinate was stronger that of glucose or citrate (Figure 3B). Among the compounds tested, the expression of the acetoin degradation genes was greatest when pyruvate or arginine were present in addition to acetoin, although the induction ratios were not as high as for glucose or citrate since the basal expression of promoter *PacoX* in the absence of acetoin was significantly higher when pyruvate or arginine were available (see Figure 3B). The repression observed in the presence of succinate, glucose or citrate was found to rely on the Crc/Hfq, Cyo and/or PTS^{Ntr} regulatory systems, although the importance of each of these elements varied according to the carbon source used.

The inhibitory effect of the Crc/Hfq regulatory proteins was detected when cells grew using succinate, glucose or citrate in addition to acetoin. Crc/Hfq had no effect, however, when pyruvate or arginine were used. The Crc/Hfq system is believed to coordinate the flow of carbon from different peripheral pathways towards the TCA cycle, helping to optimize cell metabolism and fitness (Molina et al., 2019). The metabolic signals sensed by the Crc/Hfq regulatory system are still unknown. It is likely that pyruvate and arginine do not repress the expression of the acetoin degradation genes since they are poor carbon sources for *P. putida* that support slower growth than any of the other sources tested.

A role for the Cyo regulatory system, which connects the expression of some genes to the activity of the electron transport chain, was detected when cells used succinate and acetoin, but its influence was not seen when cells were cultured with any of the other carbon sources tested. A Cyo-dependent inhibition exerted by succinate on the expression of genes for the assimilation of *n*-alkanes (Dinamarca et al., 2002, 2003) or phenol (Petruschka et al., 2001) has long been known. At least in the case of the *n*-alkanes degradation genes, Cyo was reported to have an inhibitory effect in cells cultured in LB medium. In the present work, no such effect was detected for the acetoin degradation genes, probably because the IHF-dependent 'exponential silencing' inhibition occurring in LB was so strong that it obscured the effect of any other regulatory element.

The PtsN protein, a component of the Pts^{Ntr} regulatory system, reduced acetoin's induction of the promoter *PacoX* when the cells used citrate as a carbon source, but not when they used succinate, glucose, pyruvate or arginine, or when cultured in LB medium. A glucose-dependent inhibitory effect of PtsN on the expression of the *P. putida* XylR-controlled *Pu* promoter, which is involved in the assimilation of toluene, has been recorded (Aranda-Olmedo et al., 2005, 2006; Cases et al., 1999). Under the same conditions, however, PtsN was reported not to impact the DmpR-controlled *Po* promoter involved in phenol metabolism (Sze et al., 2002). The effect of PtsN on these two promoters in cells using citrate as carbon source was not analysed, to our knowledge.

In summary, the present results support the idea that the expression of the acetoin degradation genes is carefully controlled by a combination of factors that help integrate the acetoin metabolism into the overall metabolism of the cell, allowing other carbon sources that favour faster growth to be preferentially used. Which of the global regulatory systems has the main role will depend on the compound being metabolized. The behaviour of the AcoR/*PacoX* activator/regulator pair shares similarities with, but has some differences to, other RpoN-dependent activator/promoter pairs that drive the expression of genes involved in the assimilation of non-preferred carbon sources and that are also regulated by nutritional cues. This supports the idea that there are multiple ways to correctly integrate the assimilation of non-preferred compounds into overall metabolism, thus avoiding metabolic overflows. The choice of the global regulatory system used relies as well on the activator/promoter pair involved in the induction of the genes responsible for the degradation of the non-preferred preferred compound (Shingler, 2003).

The global regulation imposed on the acetoin degradation genes favours its assimilation when it can provide an advantage to the cell. Growth at the expense of pyruvate is an example of a situation in which the co-metabolization of acetoin is advantageous. In *P. putida*, pyruvate allowed for only a very poor growth rate and did not inhibit the expression of the acetoin degradation genes. Furthermore, the simultaneous assimilation of pyruvate and acetoin led to a strong increase in growth. *Pseudomonas* can share space and resources with microorganisms that excrete pyruvate and acetoin when the consumption of sugars results in carbon overflow (Paczia et al., 2012; Xiao & Xu, 2007). In such

situations, *P. putida* could profit from the mixture of pyruvate and acetoin that remains as a leftover, becoming more competitive. These findings add to our knowledge of the array of pseudomonad fitness traits and help to explain the exceptional ability of this bacterium to grow in very different environments.

AUTHOR CONTRIBUTIONS

Renata Moreno: Conceptualization (lead); data curation (equal); formal analysis (equal); funding acquisition (supporting); investigation (equal); methodology (lead); project administration (supporting); resources (equal); supervision (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Luis Yuste:** Investigation (supporting); methodology (supporting). **Fernando Rojo:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (lead); project administration (lead); resources (lead); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

All pertinent experimental data are provided in the manuscript and in the uploaded supplementary materials.

ETHICS STATEMENT

The manuscript poses no ethical concerns; it complies to local, national and international regulations and conventions, and normal scientific ethical practices have been respected.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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