Effect of Crc and Hfq proteins on the transcription, processing, and stability of the *Pseudomonas putida* CrcZ sRNA

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ABSTRACT

In *Pseudomonas putida*, the Hfq and Crc proteins regulate the expression of many genes in response to nutritional and environmental cues, by binding to mRNAs that bear specific target motifs and inhibiting their translation. The effect of these two proteins is antagonized by the CrcZ and CrcY small RNAs (sRNAs), the levels of which vary greatly according to growth conditions. The *crcZ* and *crcY* genes are transcribed from promoters *PcrcZ* and *PcrcY*, respectively, a process that relies on the CbrB transcriptional activator and the RpoN σ factor. Here we show that *crcZ* can also be transcribed from the promoter of the immediate upstream gene, *cbrB*, a weak constitutive promoter. The *cbrB-crcZ* transcript was processed to render a sRNA very similar in size to the CrcZ produced from promoter *PcrcZ*. The processed sRNA, termed CrcZ*, was able to antagonize Hfq/Crc because, when provided in *trans*, it relieved the deregulated Hfq/Crc-dependent hyperrepressing phenotype of a Δ*crcZ*Δ*crcY* strain. CrcZ* may help in attaining basal levels of CrcZ/CrcZ* that are sufficient to protect the cell from an excessive Hfq/Crc-dependent repression. Since a functional sRNA can be produced from *PcrcZ*, an inducible strong promoter, or by cleavage of the *cbrB-crcZ* mRNA, *crcZ* can be considered a 3′-untranslated region of the *cbrB-crcZ* mRNA. In the absence of Hfq, the processed form of CrcZ was not observed. In addition, we show that Crc and Hfq increase CrcZ stability, which supports the idea that these proteins can form a complex with CrcZ and protect it from degradation by RNases.

Keywords: global regulation of gene expression; catabolite repression; small RNAs; RNA processing; bacteria

INTRODUCTION

Small RNAs (sRNAs) are key elements in controlling the expression of bacterial genomes. They act post-transcriptionally, usually to inhibit translation of a given mRNA, although some can also activate translation or bind to and regulate the activity of specific proteins (Frohlich and Vogel 2009; Bobrovskyy and Vanderpool 2013; Wagner and Romby 2015). In addition to controlling the expression of specific genes, their influence on diverse transcriptional regulators allows them to modulate important regulatory networks (Romby et al. 2006; Waters and Storz 2009; Storz et al. 2011; Mandin and Guillier 2013). sRNAs are particularly useful for controlling rapid responses to environmental or physiological signals (Wassarman 2002; Bobrovskyy and Vanderpool 2013). For a metabolically versatile bacterium that thrives in a constantly changing environment, a rapid response can be especially important to allow it to profit from transitory nutrients.

Bacteria of the genus *Pseudomonas* are a clear example in this respect. They are metabolically and physiologically very flexible and have a considerable adaptive capacity that allows them to thrive in many different environments, even in extreme conditions (Silby et al. 2011; Wu et al. 2011; Moreno and Rojo 2014). Several recently described sRNAs have key roles in controlling many aspects of *Pseudomonas* physiology (Sharma and Storz 2011; Sonnleitner and Haas 2011; Gómez-Lozano et al. 2015). One of these, CrcZ, participates in a complex regulatory network that modulates the expression of many genes involved in nutrient assimilation, thus optimizing metabolism and improving growth (Sonnleitner et al. 2009; Moreno et al. 2012). CrcZ is found in all *Pseudomonas* for which the genome sequence is available. Many *Pseudomonas* species contain, in addition to CrcZ, other very similar sRNAs that are functionally redundant, such as CrcY in *Pseudomonas putida* (Moreno et al. 2012) or CrcX in *Pseudomonas syringae* (Filiatrault et al. 2013). CrcZ

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and its sRNA homologs regulate gene expression in association with the Hfq and Crc proteins.

Hfq is a hexameric RNA-binding protein that can recognize specific targets in RNAs and has a central role in post-transcriptional gene regulation. In *Escherichia coli*, it can facilitate the annealing of sRNAs to specific mRNAs to modulate translation, influence RNA decay, and enable the assembly of some ribonucleoprotein complexes (for recent reviews, see Vogel and Luisi 2011; Sobrero and Valverde 2012; De Lay et al. 2013; Wagner and Romby 2015). In *Pseudomonas*, Hfq is a global regulator that influences diverse features such as growth, metabolism, virulence, motility, quorum sensing, and tolerance to stress (Sonnleitner et al. 2006; Sonnleitner and Blasi 2014; Arce-Rodriguez et al. 2015; Madhushani et al. 2015; Moreno et al. 2015).

The Crc protein cooperates with Hfq to form stable complexes with RNAs bearing an A-rich motif with the consensus sequence AAnAAnAA. Although Crc was initially thought to bind RNA on its own (Moreno et al. 2009; Sonnleitner et al. 2009), recent results indicate that this is not the case, but rather that Hfq is the protein that initially recognizes the A-rich motif (Sonnleitner and Blasi 2014). The Crc protein appears to stabilize the Hfq–RNA complex and forms a tripartite Hfq–RNA–Crc complex (Madhushani et al. 2015; Moreno et al. 2015). Several mRNAs that specify proteins involved in the uptake and metabolism of carbon sources, or in other physiological processes, bear A-rich Hfq targets that overlap the translation initiation site; Hfq/Crc binding to these targets inhibits translation initiation (Sonnleitner and Blasi 2014; Madhushani et al. 2015; Moreno et al. 2015).

When cells grow in nutritionally rich conditions, a situation that elicits a carbon catabolite repression (CCR) response, the repressive effect of Hfq and Crc is strong. In contrast, when the preferred nutrient-rich compounds are consumed or are lacking, the effect of Hfq/Crc is low or undetectable. Hfq and Crc levels are similar in distinct growth conditions (Moreno et al. 2015). CrcZ levels (and those of homologous sRNAs such as CrcY) vary greatly in response to nutritional cues, however, and are inversely proportional to the strength of the inhibitory effect of Hfq and Crc proteins. CrcZ and its homologous sRNAs have several A-rich motifs and can bind Hfq/Crc in vitro. It was thus suggested that these sRNAs counteract Hfq/Crc repression by sequestering one or both proteins, making them unavailable to act on mRNAs with A-rich motifs. CrcZ/CrcY levels are low in nutritionally rich conditions, which would allow Hfq/Crc proteins to repress target mRNAs. When nutrients are scarce or render low energetic return, CrcZ/CrcY levels increase and could then sequester Hfq/Crc proteins, impeding their action on target mRNAs (Sonnleitner et al. 2009; Moreno et al. 2012; Filiatrault et al. 2013; Valentini et al. 2014).

The role of the Hfq/Crc/CrcZ-CrcY regulatory system in controlling gene expression, and the underlying molecular mechanisms, have been studied in some detail in *Pseudomonas putida*, a ubiquitous soil bacterium that has become an important model system in biotechnology (Poblete-Castro et al. 2012; Nikel et al. 2014). The *P. putida crcZ* and *crcY* genes are transcribed from promoters *PcrcZ* and *PcrcY*, respectively, which require the CbrB transcriptional activator and the RNA polymerase form bound to the RpoN alternative σ factor (Moreno et al. 2012; García-Mauriño et al. 2013). The activity of these promoters varies considerably, depending on the nutritional conditions. The *crcY* gene can also be transcribed from an unknown promoter upstream of *PcrcY*. The resulting transcript is processed to render a sRNA similar in size to that of CrcY (García-Mauriño et al. 2013); the possible function of this processed transcript was not studied.

Here we show that *crcZ* is transcribed not only from *PcrcZ*, but from the promoter of the *cbrB* gene, located immediately upstream of *crcZ*. The *cbrB-crcZ* transcript is processed to render a sRNA very similar in size to the primary CrcZ produced from *PcrcZ*. This suggests that *crcZ* is a 3′-untranslated region (3′-UTR) of *cbrB* that can generate a sRNA either from *PcrcZ* or by cleavage of the *cbrB-crcZ* mRNA. We analyzed the ability of the processed form of CrcZ to control Hfq/Crc function in vivo. In addition, we show that Hfq and Crc protect the primary and processed CrcZ forms from cleavage by ribonucleases, thus increasing their stability. Our data support a model that explains how the Hfq/Crc/CrcZ regulatory system could react to nutritional signals.

RESULTS

Cells lacking RpoN contain substantial amounts of CrcZ sRNA

In agreement with reports that promoter *PcrcZ* requires the CbrB transcriptional activator and the RpoN-RNA polymerase (Moreno et al. 2012; García-Mauriño et al. 2013), the β-galactosidase activity derived from a plasmid-borne *PcrcZ-lacZ* transcriptional fusion was very low in *P. putida* cells that lacked the RpoN σ factor or the CbrB activator (Fig. 1A,B). Based on this finding, the amount of CrcZ in these mutant strains should be low or undetectable; nonetheless, real-time RT–PCR monitoring of *crcZ* transcript levels (or transcripts including *crcZ* sequences) showed that the abundance of *crcZ* transcripts in the RpoN-null strain was similar to that of the wild-type strain, both during exponential growth in LB and in the stationary phase of growth (Fig. 1C). The CbrB-null strain behaved as predicted, with very low *crcZ* transcript levels (Fig. 1C). These results suggest that, at least in the RpoN-null strain, *crcZ* can be transcribed from an RpoN-independent promoter. The DNA segment in the *PcrcZ-lacZ* transcriptional fusion, including the promoter *PcrcZ*, spanned from the end of *cbrB* to the start of *crcZ* (plasmid pPcrcZ; Fonseca et al. 2013). Since this fusion was inactive in the RpoN-null strain, *crcZ* transcription in this strain must derive from an RpoN-independent promoter located

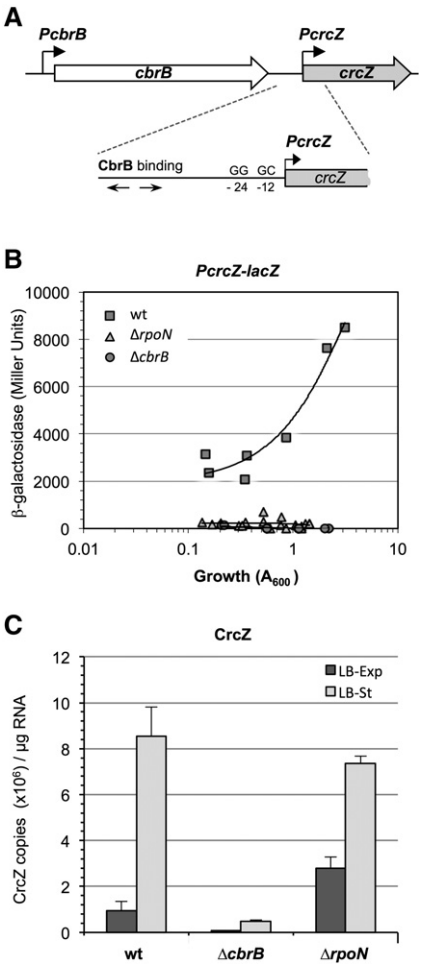

FIGURE 1. Influence of CbrB and RpoN on the activity of promoter *PcrcZ* and on CrcZ levels. (*A*) Scheme showing the location of promoter *PcrcZ*, the −12 and −24 consensus motifs for the RpoN σ factor, and the binding site for the CbrB transcriptional activator. (*B*) *P. putida* strains KT2442 (wt), MPO401 (*cbrB::km* derivative of KT2442, indicated as Δ*cbrB*), and KT2442*rpoN::Ωkm* (*rpoN::Ωkm* derivative of KT2442, indicated as Δ*rpoN*), transformed with plasmid pPcrcZ (includes a *PcrcZ-lacZ* transcriptional fusion, but lacks *cbrB* sequences), were cultured in LB medium, and β-galactosidase levels were measured at different times. The plot shows the values obtained as a function of cell growth (turbidity at 600 nm). (*C*) CrcZ levels in the above strains, cultured in LB medium and collected at mid-exponential phase (A_{600}, 0.6; dark bars) or at the start of the stationary phase (turbidity, 2.2; light bars). RNA levels were determined by real-time RT-PCR; values shown are the mean ± SD for three independent assays.

within *cbrB* or upstream of it. When sequenced, the chromosomal region spanning from the start of *cbrB* to *crcZ* in the RpoN-null strain was found to be identical to the same region in the wild-type strain. This rules out the presence of a new promoter in the RpoN-null strain that would explain the high *crcZ* transcript levels. This sequence identity also indicates that, in the wild-type strain, *crcZ* could be transcribed not only from *PcrcZ*, but also from an RpoN-independent promoter. The absence of *crcZ* transcripts in the MPO401 CbrB-null strain (García-Mauriño et al. 2013) is probably due to the deletion of almost the entire *cbrB* gene, and to the kanamycin-resistance gene that replaces the deleted *cbrB* sequences and includes a transcriptional terminator that can stop transcription originated in upstream regions.

Identification of promoters upstream of *PcrcZ* that can drive *crcZ* transcription

To assess whether *crcZ* is under the influence of a promoter located upstream of *PcrcZ*, we performed reverse transcription-PCR assays with total RNA from the wild-type and RpoN-null strains, and various primer pairs designed to detect transcripts that originate upstream of *PcrcZ* and span into *crcZ*. Results confirmed the presence of a transcript that includes *crcZ* and originates between the end of *cbrB* and *PcrcZ*, within *cbrB*, or upstream of *cbrB* (Fig. 2A).

Two parallel approaches were used to locate this promoter. We first tested whether *crcZ* can be transcribed from the *cbrB* promoter, termed *PcbrB* (Amador 2011), or from an uncharacterized internal promoter within *cbrB*. The activity of a plasmid-borne transcriptional fusion of *cbrB* (including the *PcbrB* promoter) to *lacZ* was compared to that of a series of similar reporter fusions in which the 5′-end of *cbrB* had been trimmed progressively to eliminate *PcbrB* and downstream sequences (see Fig. 2B). Deletion of promoter *PcbrB* completely eliminated reporter gene transcription (Fig. 2C), indicating that *cbrB* has no internal promoters that could affect *crcZ*, and that the *crcZ* transcripts that originate upstream of *PcrcZ* might derive from promoter *PcbrB*.

Introduction of the *cbrB-lacZ* reporter fusion contained in plasmid pFW1 (see Fig. 2B,C) into the RpoN-null strain KT2442*rpoN*::Ωkm, showed that β-galactosidase levels were three times higher in the absence of RpoN than in its presence (Fig. 2D, left panel). Real-time RT-PCR assays confirmed that transcripts for *cbrB* were twofold more abundant in the RpoN-null than in the wild-type strain (Fig. 2D, right panel). *PcbrB* promoter activity can thus explain, at least in part, the presence of *crcZ* transcripts in the RpoN-null strain. This activity was nevertheless much lower than that of promoter *PcrcZ* in the same conditions (cf. Figs. 1B, 2C).

In an alternative approach, we used 5′ RACE to test for the possible presence of a *crcZ* promoter in the vicinity of *PcrcZ* (Fig. 3A). RNA preparations from the wild-type and RpoN-null strains growing exponentially in LB medium were treated with the TEX nuclease (terminator 5′-phosphate-dependent exonuclease), which digests RNAs that have a 5′ monophosphate, but is inactive on primary transcripts with a triphosphate at their 5′ end. In the wild-type strain, the 5′-end of most of the transcripts detected (64%) corresponded to an A residue located 2 nucleotides (nt) downstream from the start site mapped by Moreno et al. (2012) and 1 nt downstream from that reported by García-Mauriño et al. (2013), both of which were determined using a primer extension approach. In the RpoN-null strain, many fewer clones

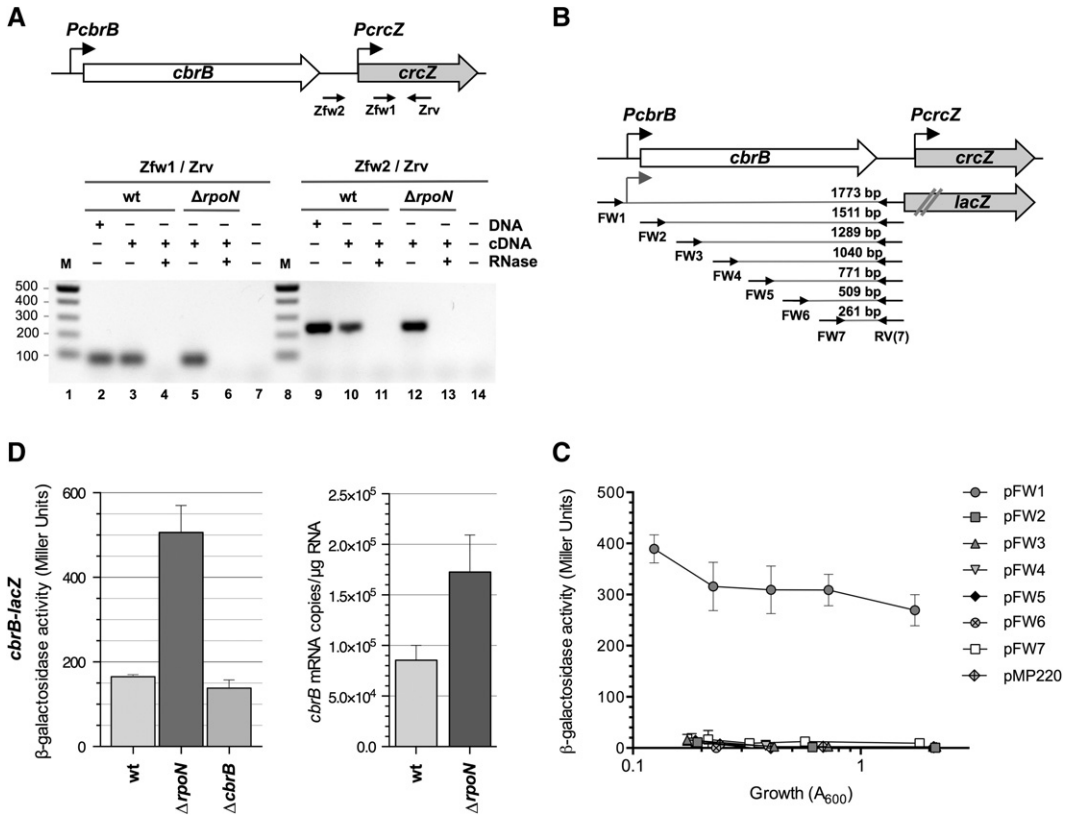

FIGURE 2. The *crcZ* gene can be transcribed from promoter *PcbrB*. (*A*) Identification of transcripts comprising *crcZ* that originate at or upstream of the promoter *PcrcZ*, detected by RT-PCR. Total RNA from the wild-type (KT2442) and RpoN-null (KT2442*rpoN*::Ω*km*) strains cultured in LB medium was purified, transformed to cDNA with reverse transcriptase and a primer that hybridized within *crcZ* (Zrv; see figure); the cDNA was amplified by PCR using forward primers that hybridized up- (Zfw2) or downstream (Zfw1) from promoter *PcrcZ*, and the reverse Zrv primer that hybridized within *crcZ*. Control reactions to which RNase A was added, or in which the PCR was performed with genomic DNA, were included as controls. (M) DNA size ladder (in bp). (*B*) Transcriptional fusions of the *cbrB* gene, or of serial deletions affecting its 5′-region, to the *lacZ* reporter gene. The *cbrB* segments fused to *lacZ* were generated by PCR with forward primers FW1 to FW7, and with the reverse primer RV(7); note that only the segment generated with primer FW1 includes the *PcbrB* promoter and that none of the fusions includes the sequences corresponding to promoter *PcrcZ* or *crcZ*. DNA segment size is indicated. (*C*) β-Galactosidase activity displayed by *P. putida* KT2440 harboring plasmids that include the transcriptional fusions constructed in *B*, named according to the corresponding forward primer. A strain bearing the plasmid vector used to construct the fusions (pMP220) was included as control. Cells were cultured in LB medium, samples were withdrawn at various times, and β-galactosidase activity was determined using ONPG as the substrate; data shown are the mean ± SD for three independent assays. (*D*) Influence of RpoN on *cbrB* gene expression. The *left* panel shows the transcription of *cbrB*, as deduced from a *cbrB-lacZ* transcriptional fusion, in *P. putida* strains KT2442 (wt) and KT2442*rpoN*::Ω*km* (Δ*rpoN*), transformed with plasmid pFW1 (contains the *cbrB-lacZ* transcriptional fusion; see panel *B*). Cells were cultured in LB medium; at mid-exponential phase (A_{600}, 0.6), samples were taken and β-galactosidase activity was determined using ONPG as substrate. Data shown are the mean ± SD for three independent assays. The *right* panel shows the abundance of *cbrB* transcripts in *P. putida* strains KT2442 (wt) and KT2442*rpoN*::Ω*km* (Δ*rpoN*), determined by real-time RT-PCR with RNA from cells cultured in LB medium and collected at mid-exponential phase (A_{600}, 0.6). The primers used for the PCR reaction hybridized at the central region of *cbrB*. Values show the mean ± SD for three independent assays.

were isolated from the RACE assays, which suggests the absence of primary *crcZ* transcripts. In addition, the 5′-end indicated by the isolated clones mapped in most cases at positions 5–14 nt upstream of the +1 start site observed for CrcZ in the wild-type strain; 50% of these 5′-ends mapped at positions −5 and −6 (Fig. 3A). The heterogeneity of the 5′-ends suggests that they are processed transcripts that escaped the TEX treatment.

The 5′ end of *crcZ* transcripts was also analyzed using primer extension assays performed with an end-labeled oligonucleotide primer. When using TEX-treated RNA samples, we detected an extension product in the wild-type strain, which corresponded to a transcript originated at the transcription start site detected in the RACE assays (position +1; Fig. 3B). This extension product was barely visible in the RNA sample from the RpoN-null strain (Fig. 3B). In the absence of TEX treatment, most extension products in the RpoN-null strain samples were transcripts with 5′ ends matching positions −6, −9, −11, or −13. Within the accuracy limits of the primer extension assays, these 5′ ends coincide with those found using 5′ RACE assays. The extension products with 5′ ends in the −6 to −13 region were also detected in the TEX-untreated wild-type strain samples, although their abundance was much lower than in the RpoN-null strain and represented a minor fraction of overall *crcZ* transcripts (Fig. 3B). These results support the idea that promoter

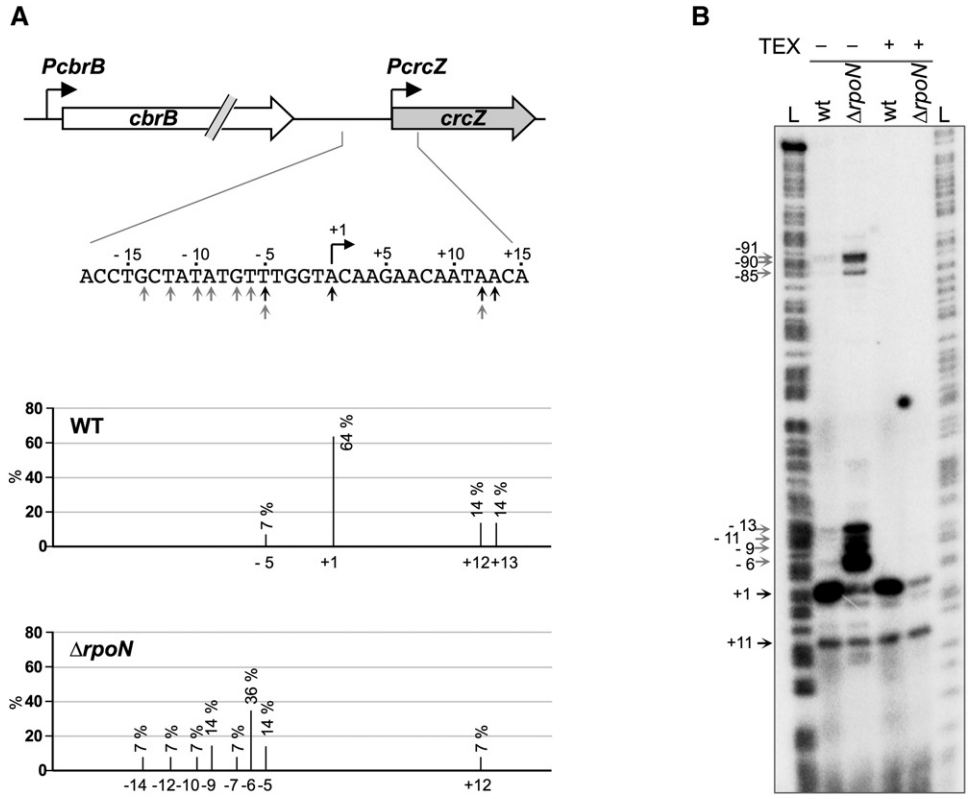

FIGURE 3. Identification of the 5′ end of *crcZ* transcripts in wild-type and *rpoN P. putida* strains. Total RNA was obtained from *P. putida* KT2442 (wt) and KT2442*rpoN::Ωkm* (Δ*rpoN*) cultured in LB medium and the 5′ end of the *crcZ* transcripts were determined using 5′ RACE (*A*) or primer extension (*B*). (*A*) 5′ RACE assays were performed with RNA samples previously treated with terminator 5′-phosphate-dependent exonuclease (TEX). Fourteen independent clones were sequenced for each strain; the 5′ ends found for the wild type are indicated by black arrows, and those for the *rpoN* strain by gray arrows. The frequency with which each 5′ end was found is indicated in the *lower* panels (in %). (*B*) Primer extension reactions were performed with an end-labeled oligonucleotide and RNA samples, untreated or treated with TEX enzyme, as indicated. The extended cDNA products were analyzed by electrophoresis on a denaturing 6% urea–polyacrylamide gel, in parallel with a DNA sequence ladder (L). Numbers (*right*) indicate the position of the 5′ ends detected relative to the *crcZ* transcription start site (+1).

PcrcZ is inactive in the absence of RpoN and that additional promoters cannot be detected in this region. The transcripts detected in the RpoN-null strain are probably due to read-through transcription from *cbrB* and cleavage of the *cbrB-crcZ* transcripts.

The transcript originated at *PcbrB* can be processed to generate an sRNA similar to the primary CrcZ

When we used a probe for *crcZ*, Northern blot analysis of RNA samples from cells growing exponentially in LB medium showed that the RpoN-null strain bears a transcript that hybridizes to the probe and is very similar in size to CrcZ (Fig. 4). This transcript was barely detected in TEX-treated samples, which indicates that it is not a primary transcript, but derives from processing of a larger transcript that probably originated at the *PcbrB* promoter. This idea coincides with the results of the 5′ RACE and primer extension assays for the RpoN-null strain (Fig. 3), which suggest a processing site located 5–9 nt upstream of the promoter *PcrcZ* +1 start site. For simplicity, these processed transcripts of very similar size were collectively termed CrcZ*. In the wild-type strain, however, most *crcZ* transcripts were TEX-resistant, although quantitation of the bands in three independent assays indicated that transcript levels decreased by ∼20% after TEX treatment. This again suggests that processed CrcZ* transcripts are also present in the wild-type strain.

Real-time RT-PCR assays performed with RNA from cells growing exponentially in LB medium showed that the number of transcripts that include *crcZ* sequences were much more abundant than those for *cbrB* (∼10-fold for the wild-type strain and 16-fold for the RpoN-null strain; compare data in Fig. 1C and Fig. 2D). This suggests that, after cleavage of the *cbrB-crcZ* transcript, the 5′-segment for *cbrB* is rapidly degraded, while the fragment corresponding to *crcZ* is more stable and accumulates, particularly in the RpoN-null strain.

In the CbrB-null strain MPO401, neither the primary nor the processed *crcZ* transcripts were detected (Fig. 4). Primary transcripts that originated at *PcrcZ* are predicted to be absent in this strain, since this promoter relies on the CbrB activator, which is lacking. The absence of processed *crcZ* transcripts is probably the consequence of the kanamycin-resistance

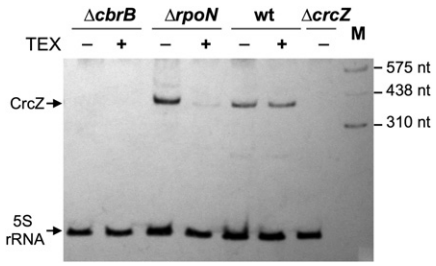

FIGURE 4. Identification of CrcZ sRNA by Northern blot in strains KT2442 (wt), MPO401 (Δ*cbrB*), KT2442*rpoN::Ωkm* (Δ*rpoN*). Strain KT2440-Z, which has an inactivated *crcZ::tet* allele and lacks CrcZ, is included as control (indicated as Δ*crcZ*). Total RNA was obtained from cells growing exponentially in LB medium (A_{600}, 0.6). RNA samples were untreated or treated with TEX, resolved on a 6% polyacrylamide/7 M urea gel, transferred to a nitrocellulose membrane, and the presence of CrcZ and 5S rRNA determined by hybridization with specific probes. Lane *M* corresponds to an RNA size ladder.

determinant that interrupts the *cbrB* gene in this strain, and stops transcripts that originate at promoter *PcbrB*.

The processed form of CrcZ can antagonize Hfq/Crc function, complementing the hyperrepressing phenotype of a Δ*crcZ*Δ*crcY* double mutant

Our results showed that cells can bear two CrcZ variants, the primary CrcZ and the processed CrcZ*. The processed form is the predominant, if not the only variant in the RpoN-null strain, whereas most CrcZ in the wild-type strain corresponds to the primary transcript. Prediction of the secondary structure of CrcZ* and CrcZ, using the RNAfold software package (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi), suggested that the overall structure of these sRNAs is highly similar. Although there are some local modifications due to the extra nucleotides present at the CrcZ* 5′-end, they are small, and the stem–loops that include the Hfq binding sites remain unchanged (not shown). We thus assessed whether the processed CrcZ* variant is functional and can antagonize the repressing activity of Hfq/Crc proteins, which would open up a possible role for CrcZ* in the CbrB/Hfq/Crc regulatory system. We previously reported that a mutant *P. putida* strain bearing inactivated *crcZ* and *crcY* genes, and therefore lacking all forms of CrcZ and CrcY, is unable to grow in a minimal salts medium containing benzoate or citrate as the sole carbon source, and it has a markedly reduced growth rate when glucose, fumarate, or succinate are provided as the carbon source (Moreno et al. 2012). Since the wild-type strain can grow efficiently using any of these compounds, we proposed that lack of CrcZ and CrcY (and of its processed forms, CrcZ* and CrcY*) deregulates the Hfq/Crc system, leading to strong, constitutive Hfq/Crc-dependent repression of genes involved in the uptake and assimilation of these nonpreferred carbon sources (Moreno et al. 2012).

To test whether CrcZ* expression in *trans* complements the Δ*crcZ*Δ*crcY* strain growth defects, two plasmids were constructed. The first, p421-Pwt, bore a DNA segment that includes the *cbrB* and *crcZ* genes (expressed from their native promoters, *PcbrB* and *PcrcZ*) and can therefore generate CrcZ and CrcZ* (see Fig. 5A). The second plasmid, p421-Pmut, has a variant of this DNA fragment in which the GG and GC nucleotides at the consensus −24 and −12 regions of promoter *PcrcZ* were modified to CC and TT, respectively. This was predicted to inactivate promoter *PcrcZ*, such that plasmid p421-Pmut would generate the processed CrcZ* form but not the primary CrcZ transcript. To verify that the mutations inactivated *PcrcZ*, a DNA segment from p421-Pmut spanning from the end of *cbrB* to the promoter *PcrcZ* start site was PCR amplified and cloned into the pMP220 reporter plasmid, generating a transcriptional fusion to the *lacZ* indicator gene. The plasmid obtained, pPcrcZmut, was introduced into strain KT2440, and its ability to produce β-galactosidase was compared to that of plasmid pPcrcZ, which bears a wild-type *PcrcZ* promoter. As predicted, the mutant promoter was totally inactive (Fig. 5B).

We introduced plasmids p421-Pwt and p421-Pmut into the Δ*crcZ*Δ*crcY* strain KT2440-ZY and analyzed their ability to produce CrcZ and/or CrcZ*. As control, the empty plasmid vector (pSEVA421) was also introduced into strain KT2440-ZY. Northern blot analysis showed that plasmid p421-Pwt allowed production of an sRNA that hybridizes to the CrcZ probe and whose abundance decreases little after TEX treatment (Fig. 5C). This suggests that most sRNA produced is primary CrcZ, although CrcZ* would also be present. In contrast, the sRNA species produced by plasmid p421-Pmut was completely degraded by TEX treatment, indicating that this plasmid generates CrcZ* but not primary CrcZ. Real-time RT-PCR indicated that, in cells growing exponentially in LB medium, the amount of CrcZ* in the mutant strain KT2440-ZY containing p421-Pmut was similar to that of CrcZ in the wild-type KT2440 strain (Fig. 5D); nonetheless, the amount of CrcZ produced from plasmid p421-Pwt in strain KT2440-ZY was twice as high.

We next analyzed the ability of plasmids p421-Pwt and p421-Pmut to restore the growth defect of strain KT2440-ZY in a minimal salts medium containing citrate, benzoate, glucose, or succinate as the sole carbon source. When using citrate or benzoate, the control strain KT2440-ZY (pSEVA421) could not grow even after 24 h in culture, while the wild-type strain KT2440(pSEVA421) grew efficiently (Fig. 6). The presence of plasmid p421-Pwt in the CrcZ/CrcY-null strain KT2440-ZY fully restored growth, with a growth rate similar to that of the wild-type KT2440 strain. This suggests that the CrcZ and CrcZ* sRNAs produced from plasmid p421-Pwt can complement the growth defect of strain KT2440-ZY when citrate or benzoate are the only carbon source available. Plasmid p421-Pmut, which generates CrcZ* but not the primary CrcZ (see Fig. 5C), provided partial complementation, since cells grew slowly and cultures eventually reached turbidity values similar to those of the wild-type strain after 24 h culture (Fig. 6). This suggests

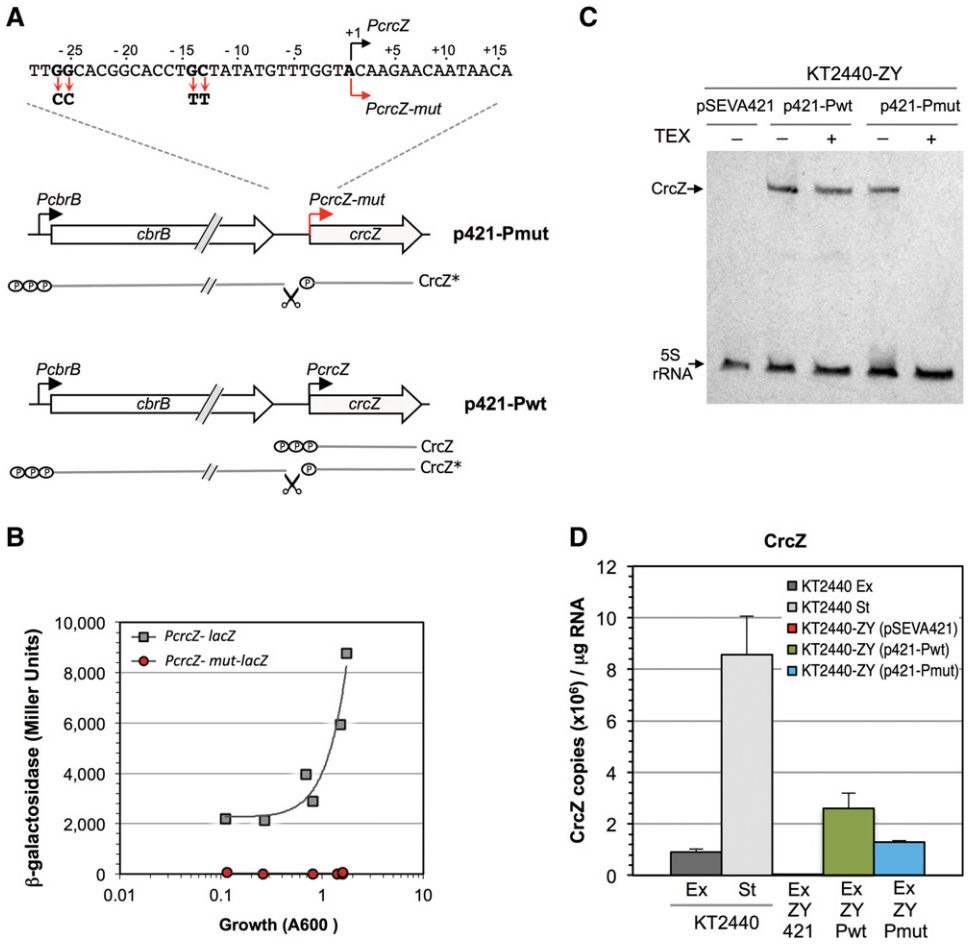

FIGURE 5. Plasmids designed to produce CrcZ and CrcZ*, or only CrcZ*. (*A*) Scheme of the insert in plasmids p421-Pwt and p421-Pmut. Plasmid p421-Pwt bears the *cbrB* and *crcZ* genes. Plasmid p421-Pmut contains *cbrB* and a mutant variant of *PcrcZ* in which the GG and GC nucleotides of the −24 and −12 regions, critical for recognition by RpoN-RNA polymerase, were modified (indicated in the blow-up). The RNAs predicted in each case are indicated. (*B*) *P. putida* strain KT2440 containing plasmid pPcrcZ (includes a *PcrcZ-lacZ* transcriptional fusion) or pPcrcZmut (includes a similar transcriptional fusion, with the mutant *PcrcZmut* promoter described in *A*), were cultured in LB medium and β-galactosidase levels measured at different times. The plot shows values obtained as a function of cell growth. (*C*) Northern blot identification of CrcZ/CrcZ* in strain KT2440-ZY containing plasmids pSEVA421 (control), p421-Pwt, or p421-Pmut. Total RNA from cells growing exponentially in LB medium was untreated or treated with TEX, resolved on a denaturing polyacrylamide/urea gel, transferred to a nitrocellulose membrane, and the presence of CrcZ and 5S rRNA determined by hybridization with specific probes. (*D*) The amount of CrcZ/CrcZ*, determined by real-time RT-PCR, in strains KT2440 and KT2440-ZY containing the plasmids is indicated in *C*; total RNA was purified from cells cultured in LB medium and collected at mid-exponential phase (A_{600} of 0.6; "Ex") or in stationary phase (A_{600} of 2.2; "St").

that CrcZ* can at least partially antagonize the inhibitory effect of Hfq/Crc. Most likely, complementation is only partial because the amount of CrcZ* produced from the weak *PcbrB* promoter is lower than that of CrcZ generated from the strong *PcrcZ* promoter.

Lack of CrcZ and CrcY had a less severe effect when strain KT2440-ZY used glucose or succinate as the carbon source; cells grew slowly, although after 24 h, turbidity values were high (Fig. 6). Plasmid p421-Pmut could effectively complement the lack of CrcZ and CrcY, particularly when succinate was the carbon source (Fig. 6). Since this plasmid allows production of CrcZ*, but not of the primary CrcZ, its ability to restore the growth of strain KT2440-ZY on glucose or succinate further shows that the processed CrcZ* form can at least partially antagonize the repressive effect of the Hfq/Crc proteins, supporting that it is functional.

Since the amount of CrcZ* present in a wild-type strain is low, it can be asked whether it plays an active role in the modulation of Hfq/Crc-dependent catabolite repression when growth conditions change from a situation of high repression (for example, a complete medium) to one of low repression (a poor carbon source available). We therefore analyzed whether the amount of CrcZ* produced from promoter *PcbrB* is high enough to relieve the Hfq/Crc-dependent repression of the *benA* gene, which codes for the first enzyme of the benzoate assimilation pathway. Transcription of *benA* requires the BenR transcriptional activator. Earlier work showed that the Hfq/Crc system inhibits translation

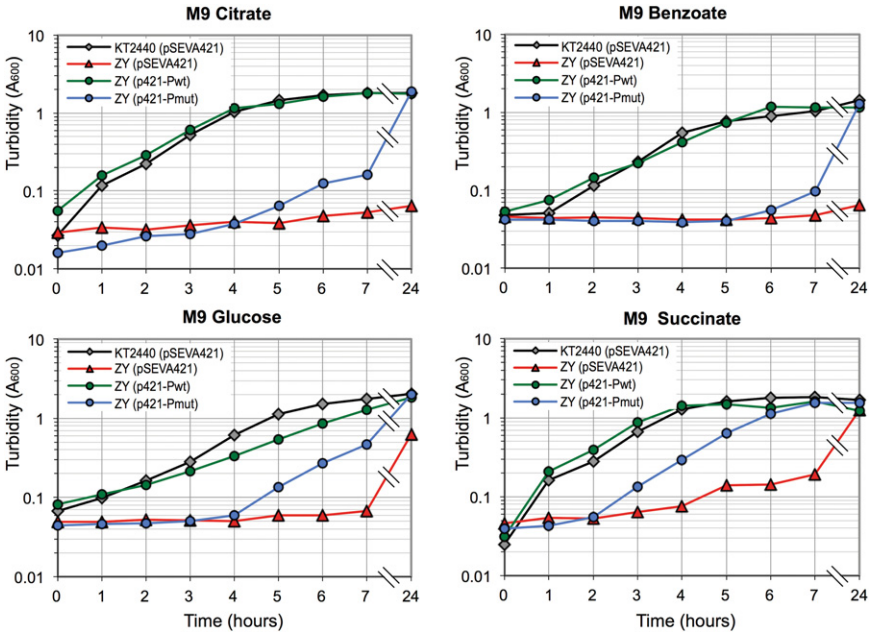

FIGURE 6. Growth of *P. putida* strains KT2440 (wild type) and KT2440-ZY (a Δ*crcZ*Δ*crcY* derivative of strain KT2440), containing plasmids pSEVA421 (vector with no insert), p421-Pwt (produces CrcZ and CrcZ*), or p421-Pmut (produces CrcZ*), in M9 minimal salts medium containing citrate, benzoate, glucose, or succinate as sole carbon source. Note discontinuity in the time scale after hour 7.

of *benR* mRNA, which bears an A-rich motif to which Hfq/Crc bind (Moreno and Rojo 2008; Hernández-Arranz et al. 2013). As a result, in cells growing exponentially in benzoate-containing LB medium, Hfq/Crc keep BenR levels below those needed for an efficient induction of the *benA* gene, exerting an indirect but strong inhibition of *benA* transcription (Morales et al. 2004; Moreno and Rojo 2008; Moreno et al. 2012; Hernández-Arranz et al. 2013).

The indirect Hfq/Crc-dependent inhibition of *benA* transcription can be monitored using *P. putida* strain PBA1, which bears a *PbenA-lacZ* transcriptional fusion in its chromosome, and its derivatives PBA1C (Crc-null) and PBAZY (CrcZ-null, CrcY-null; lacking all forms of these sRNAs) (Moreno and Rojo 2008; Moreno et al. 2012). Plasmids p421-Pwt and p421-Pmut were introduced into strain PBAZY, while the empty plasmid vector pSEVA421 was introduced into strains PBA1, PBAZY, and PBA1C, as controls. The ability of benzoate to induce transcription from promoter *PbenA* was followed in all strains by measuring β-galactosidase production in cells cultured in LB medium with 5 mM benzoate. β-Galactosidase expression in strain PBA1 (pSEVA421) was strongly inhibited during the exponential growth phase until the culture reached a turbidity of ~1–1.5, when cells prepared to enter the stationary phase and Hfq/Crc-dependent repression was relieved (see Fig. 7), a process that coincides with a strong increase in CrcZ/CrcY levels (Moreno et al. 2012). Repression was not observed in the Crc-null strain, as predicted. In strain PBAZY, however, repression was strong and did not disappear after entry into stationary phase, as would be anticipated for a strain with a deregulated, constitutively active Hfq/Crc repression system. Introduction of plasmid p421-Pwt into strain PBAZY partially relieved repression, probably because the plasmid provides several copies of the *cbrB* and *crcZ* genes that give rise to increased CrcZ dosage (see Fig. 5D). The presence of p421-Pmut in strain PBAZY had no apparent effect. This plasmid allows production of CrcZ* but not of CrcZ. Since the results presented in Figure 6 had indicated that CrcZ* can at least partially antagonize the inhibitory effect of Hfq/Crc, the observation that it cannot relieve the Hfq/Crc-dependent inhibition of *benA* transcription suggests that the levels of CrcZ* produced from plasmid p421-Pmut are not high enough to counteract the Hfq/Crc-dependent inhibition of *benA* expression. In support of this idea, real-time RT-PCR assays (Fig. 5D) indicated that CrcZ* levels in a Δ*crcZ*Δ*crcY* strain containing plasmid p421-Pmut and growing exponentially in LB medium are about half those of CrcZ + CrcZ* generated from p421-Pwt, and very similar to those seen in the wild-type strain, levels that allow a strong Hfq/Crc-dependent repression. In addition, since plasmid p421-Pmut generates CrcZ* from promoter *PcbrB*, which is weak and constitutive, the amount of CrcZ* cannot increase in the stationary phase, and therefore Hfq/Crc-dependent repression of promoter *PbenA* is not relieved in the stationary phase, in contrast to what is observed when the primary CrcZ is expressed from the strong and inducible *PcrcZ* promoter. Altogether, the results presented suggest that the amounts of CrcZ* present in the cell are not sufficient to counteract the strong CCR effect that occurs in a complete medium, but can contribute to provide a basal protection from an excessive Hfq/Crc-dependent repression.

Influence of Crc and Hfq proteins on CrcZ expression, stability, and processing

P. putida CrcZ levels decrease considerably following inactivation of the *crc* gene (García-Mauriño et al. 2013). This effect has been traced to a reduction in promoter *PcrcZ* activity when the Crc protein is absent. Although the underlying reasons are unclear, the authors proposed that Crc inhibits translation of an mRNA encoding a transcriptional repressor that inhibits *PcrcZ* activity. The effect of Hfq on CrcZ levels and *PcrcZ* activity was unknown. Northern blot analysis showed that lack of Hfq also led to a marked decrease in CrcZ levels (Fig. 8A), which was confirmed by real-time RT-PCR (not shown). The absence of Hfq also had a negative

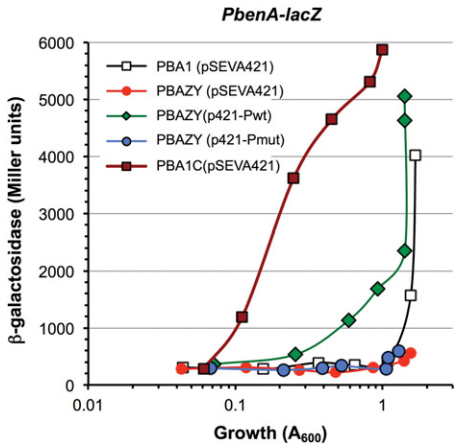

FIGURE 7. *P. putida* strains PBA1 (contains a *PbenA-lacZ* transcriptional fusion in its chromosome), PBA1C (a Crc-null derivative of strain PBA1), or PBAZY (a Δ*crcZ*Δ*crcY* derivative of strain PBA1), where indicated containing plasmids pSEVA421, p421-Pwt, or p421-Pmut, were cultured in LB medium with 5 mM benzoate, and β-galactosidase levels were measured at different times. The plot shows the values as a function of cell growth (turbidity at 600 nm). Exponential growth declined at a turbidity of ~1.5, when cells prepared to enter the stationary growth phase.

effect on promoter *PcrcZ* activity (Fig. 8B). The strong decrease in CrcZ when Crc or Hfq proteins are absent might nonetheless be due not only to reduced *PcrcZ* activity, but also to reduced sRNA stability, since the CrcZ sRNA has several A-rich motifs that are very similar to RNase E targets.

As Hfq/Crc binding to CrcZ might protect the sRNA from RNase degradation, which could affect CrcZ levels, we analyzed the influence of Crc and Hfq on CrcZ stability. Since CrcZ levels in cells lacking Crc or Hfq are essentially undetectable, we introduced plasmid p424-Z, which allows *crcZ* transcription from the heterologous IPTG-inducible *Ptrc* promoter, into *P. putida* strains KT2440Δ*hfq* (Hfq-null) and KTCRC (Crc-null). As a control, the same plasmid was introduced into the CrcZ-null strain KT2440-Z, which is wild type for Crc and Hfq and in which all CrcZ must be generated from plasmid p424-Z. The three strains were cultured in LB medium with 1 mM IPTG (to induce *crcZ* transcription); cells were collected at mid-exponential phase, their RNA extracted, and CrcZ levels were analyzed by Northern blot and real-time RT-PCR. In the strain bearing wild-type *crc* and *hfq* alleles, CrcZ overproduction led to the appearance of two similarly sized bands that hybridized to the probe (Fig. 9A; lanes labeled "Δ*crcZ*"). The larger band was TEX-resistant and probably corresponds to a primary, plasmid-produced *crcZ* transcript that contains 62 extra nucleotides at its 5′-end, which is the distance between the *Ptrc* promoter transcription start site and the first *crcZ* nucleotide. The smaller band was similar in size to the CrcZ sRNA in the wild-type KT2440 strain (Fig. 9A, labelled "wt") and was selectively degraded by TEX. This smaller band probably corresponds to a processed transcript that has lost most or all of the 62-nt extra tail at the 5′ end. This processed transcript was not observed in the Hfq-null strain. This suggests that Hfq facilitates this processing or, alternatively, that Hfq protects CrcZ from degradation by RNases, protection that would be more important for the processed transcript than for the primary transcript. Although the processed *crcZ* transcript was detected in the Crc-null strain, it was somewhat smaller than in the wild-type strain (Fig. 9A), which suggests that Crc influences the structure of the RNA–Hfq complex that facilitates processing and favors a defined excision site. Real-time RT-PCR assays to quantify the combined abundance of primary and processed CrcZ forms showed that, when CrcZ was overproduced by the plasmid, lack of Crc or Hfq reduced CrcZ steady-state levels by 2.5- and 3.7-fold, respectively (Fig. 9B), which coincides with Northern blot assay results.

To analyze the influence of Crc and Hfq on the stability of *crcZ* transcripts originated from plasmid p424-Z, *P. putida* strains KT2440-Z (CrcZ-null), KT2440Δ*hfq* (Hfq-null), and KTCRC (Crc-null), all bearing plasmid p424-Z, were cultured in LB medium with IPTG, and transcription was blocked at mid-exponential phase by rifampicin addition. Samples were taken at different times, total RNA was purified from the cells, and *crcZ* transcript abundance was analyzed in

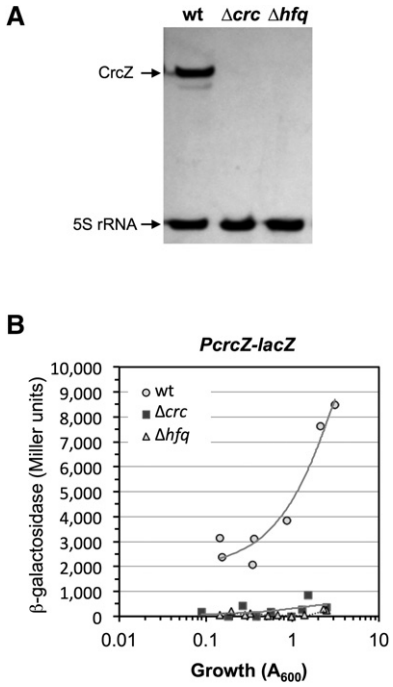

FIGURE 8. Amounts of CrcZ (*A*) and *PcrcZ* promoter activity (*B*) in *P. putida* strains KT2440 (wt), KTCRC (Δ*crc*), and KT2440Δ*hfq* (Δ*hfq* derivative of KT2440). (*A*) Total RNA was obtained from cells growing exponentially in LB medium (A_{600}, 0.6), and CrcZ and 5S rRNA determined by Northern blot with specific probes. (*B*) The strains indicated above, transformed with plasmid pPcrcZ (which bears a *PcrcZ-lacZ* transcriptional fusion), were cultured in LB medium. Samples were taken at different times and β-galactosidase levels measured using ONPG as substrate. Two independent assays were performed; results from both of them are shown.

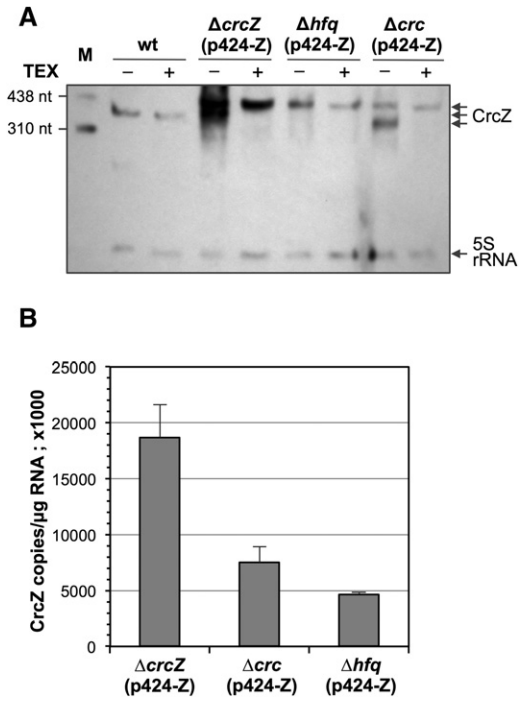

FIGURE 9. Overproduction of CrcZ in cells with wild-type, CrcZ-null, Hfq-null, or Crc-null genetic backgrounds. Plasmid p424-Z, which allows *crcZ* expression from the IPTG-inducible *Ptrc* promoter, was introduced into *P. putida* strains KT2440-Z (Δ*crcZ*), KT2440Δ*hfq* (Δ*hfq*), and KTCRC (a Δ*crc* derivative of KT2440). Cells were cultured in LB medium with streptomycin and 1 mM IPTG. At mid-exponential phase (A_{600}, 0.6), cells were collected and total RNA obtained. A wild-type KT2440 strain lacking plasmid p424-Z was cultured and processed in parallel. (*A*) RNA samples were untreated or TEX-treated, and CrcZ was analyzed by Northern blot as in Figure 4. Lane *M* shows an RNA size ladder. (*B*) The amount of CrcZ in RNA samples from strains bearing plasmid p424-Z, determined by real-time RT-PCR. Values show the mean ± SD for three independent assays.

each sample by Northern blot. The half-life of the primary *crcZ* transcript produced from the plasmid *Ptrc* promoter was ~6 min in the strain containing both Crc and Hfq (strain Δ*crcZ*), but decreased to ~3.8 min in the strain lacking Hfq, and to ~3 min when Crc was absent (Fig. 10A–C; summarized in Fig. 10D). The absence of Crc and Hfq thus reduces primary CrcZ stability by almost half. The pattern was similar for the processed *crcZ* transcript (higher electrophoretic mobility band in Northern blots); the absence of Crc reduced transcript half-life by almost half (from 6.8 to 3.8 min; Fig. 10E). It is worth noting that when Crc and Hfq proteins were both present, decay of the primary and the processed *crcZ* transcripts was delayed for ~4 min, after which their levels began to diminish. In the absence of Crc or Hfq, RNA decay began immediately.

DISCUSSION

Many sRNAs originate from noncoding genes located within intergenic regions, between protein-coding genes (Miyakoshi et al. 2015). The CrcZ sRNA, present in all Pseudomonads for which the genome sequence is known, was thought to belong to this group, since it originates from a promoter (*PcrcZ*) located downstream from the *cbrB* gene. Here we show that *crcZ* can also be transcribed from the *cbrB* promoter, which generates a *cbrB-crcZ* transcript that is processed to render a sRNA very similar in size to that of the primary CrcZ produced from promoter *PcrcZ*. Processing occurred preferentially at a site 5–9 nt upstream of the 5′-end of the primary CrcZ. In a wild-type strain, these processed variants, collectively termed CrcZ*, account for a small percentage of the total CrcZ species. In the RpoN-null strain analyzed in this work, promoter *PcrcZ* is inactive and CrcZ* is the only species present, reaching levels similar to or higher than those of the primary CrcZ in the wild-type strain. The *crcZ* gene can thus be considered a 3′-untranslated region (3′-UTR) of *cbrB* that can generate a sRNA from *PcrcZ* or by cleavage of the *cbrB* mRNA 3′-UTR (see Fig. 11). Although these two sRNA variants have different 5′-ends, they share the transcriptional terminator at the *crcZ* 3′-end.

We present evidence that an artificial CrcZ variant with an unrelated 62-nt extra tail at its 5′-end is also processed to render a sRNA similar in size to the primary CrcZ (Figs. 9, 10). This finding suggests that processing depends on a sequence or structure downstream from the cleavage site, and that the upstream sequences are less important. A similar observation was recently made for the *E. coli* GlmZ sRNA, in which a central stem–loop is decisive for cleavage by RNase E, whereas the precise sequence at the cleavage site was not important (Gopel et al. 2016). We did not detect processing of the CrcZ variant with 62 extra nucleotides at its 5′ end in the Hfq-null strain (Fig. 9), which implies that Hfq is involved in this processing or is needed to avoid transcript degradation by RNases. The *Vibrio cholerae* Hfq protein was similarly observed to participate in MicX transcript processing by RNaseE to generate a stable, more active sRNA (Davis and Waldor 2007).

In all *Pseudomonas* genomes analyzed to date, *crcZ* maps downstream from *cbrB* (Sonnleitner et al. 2009; Moreno et al. 2012; Filiatrault et al. 2013). Our finding that *cbrB* and *crcZ* are functionally linked and form a single transcriptional unit in *P. putida* suggests that *cbrB-crcZ* cotranscription, and possibly its processing to yield CrcZ*, might occur in other *Pseudomonas* species as well. The *P. putida* CrcY sRNA, which is very similar to CrcZ and functionally redundant, maps at a site distant from *crcZ*, downstream from the *mvaB* gene (Moreno et al. 2012). Like *crcZ*, *crcY* is transcribed from the CbrB-dependent *PcrcY* promoter, but transcripts that originate upstream of *mvaB* can run into *crcY*. This generates a transcript that can be processed to render sRNA similar in size to the primary CrcY generated from *PcrcY* (García-Mauriño et al. 2013). Therefore, CrcY can also be considered a 3′-UTR of *mvaB*. *Pseudomonas syringae* CrcZ and CrcX sRNAs are also proposed to arise from two promoters, one recognized by RpoN and the other by a different

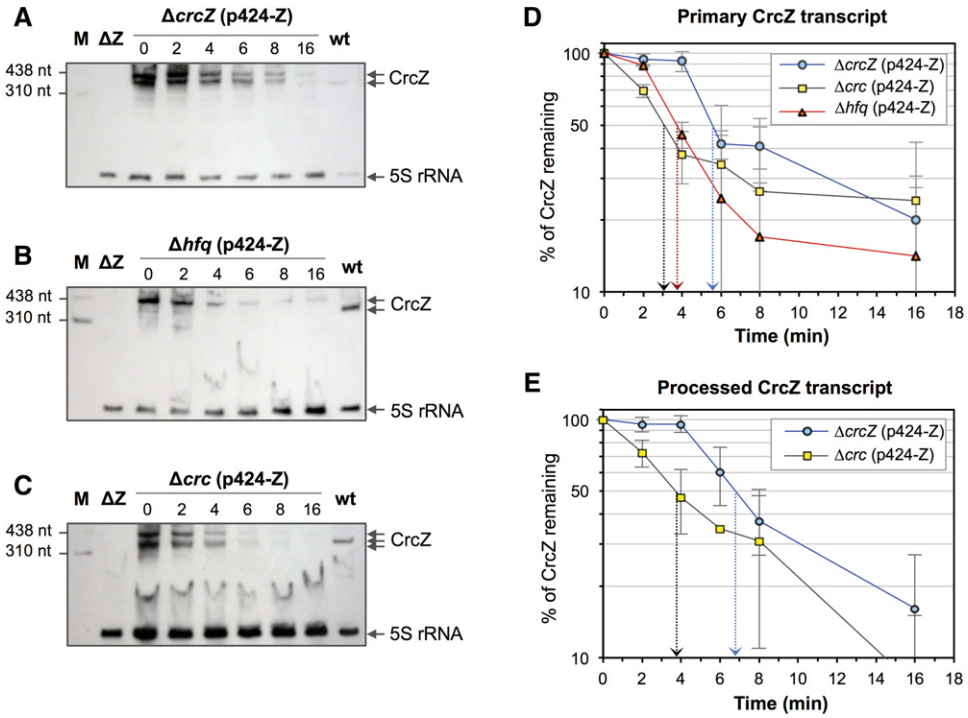

FIGURE 10. Half-life of CrcZ produced from plasmid p424-Z in cells with CrcZ-null, Hfq-null, or Crc-null genetic backgrounds. (*A*–*C*) *P. putida* strains KT2440-Z (Δ*crcZ*), KT2440Δ*hfq* (Δ*hfq*), and KTCRC (Δ*crc*) were cultured in LB medium with streptomycin and 1 mM IPTG. At a turbidity of 0.6 (A_{600}), rifampicin was added to stop transcription. Aliquots were withdrawn at 0, 2, 4, 8, 16, and 32 min post-rifampicin addition, total RNA was isolated, resolved on a 6% polyacrylamide/7 M urea gel, and CrcZ and 5S rRNA detected by Northern blot with specific probes. Band intensities were quantified using a ChemiDoc XRS imager and Image Lab software (Bio-Rad). Values obtained for the larger (primary CrcZ transcript) or shorter bands (processed CrcZ transcript) detected with the CrcZ probe were normalized to those of the 5S rRNA of the corresponding sample. (*D*,*E*) Plots show the values for each time point expressed relative to that at time 0. Processed CrcZ was not detected in the Δ*hfq* strain. Data shown as mean ± SD for three independent assays.

σ factor (Filiatrault et al. 2013). The coexistence of two forms of these sRNAs, a primary and a processed transcript of very similar sizes, could thus be common in *Pseudomonas*. Nesting a sRNA within a functionally related mRNA, with which it shares the intrinsic terminator, allows coupling a protein (a regulator) and a noncoding function (a sRNA) into a single expression unit that can be transferred horizontally to other bacteria (Miyakoshi et al. 2015).

There is evidence to support the idea that CrcZ* is a functional sRNA, able to antagonize the effect of Hfq and Crc. The growth of a Δ*crcZ*Δ*crcY* strain that lacks all forms of CrcZ and CrcY is strongly impaired when nonpreferred compounds such as citrate, benzoate, glucose, or succinate are provided as the sole carbon source in a minimal salts medium. This growth defect most likely derives from a hyperrepressing effect of the Hfq and Crc proteins on functions necessary to assimilate these compounds, an effect that cannot be controlled because CrcZ/CrcY and their processed variants are absent. Introduction of a plasmid that produces CrcZ*, but not CrcZ, into the Δ*crcZ*Δ*crcY* strain partially complemented the hyperrepressing phenotype, allowing growth on the

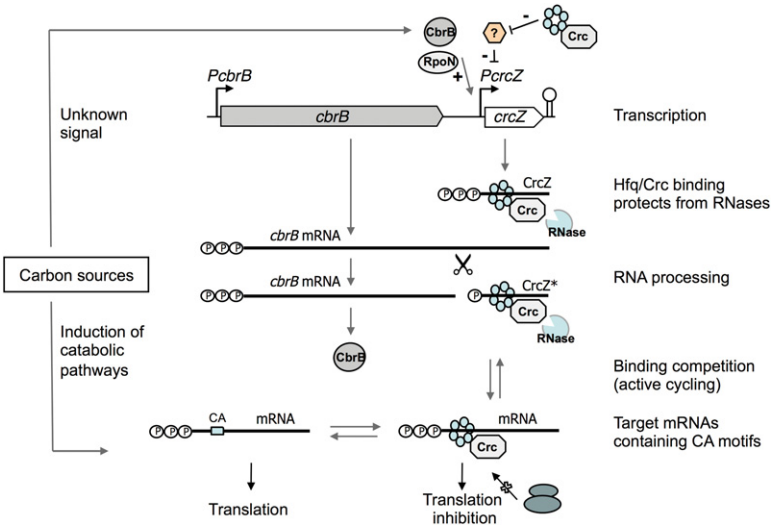

FIGURE 11. Model for the Hfq/Crc/CrcZ regulatory system. See Discussion for details.

mentioned nonpreferred compounds. This suggests that CrcZ* is functional and can antagonize the inhibitory effect of Hfq/Crc. Additional evidence that modifications at the 5′-end of CrcZ do not necessarily impair its ability to counteract Hfq/Crc activity derives from the observation that the individual overproduction of CrcZ or CrcY variants with 62 extra nt at the 5′-end, using an inducible heterologous promoter, reduces Hfq/Crc-dependent CCR in vivo (Moreno et al. 2012). This suggests that either the unprocessed 62-nt larger variants or their processed derivatives (see Fig. 9) are functional.

Although the amounts of CrcZ* provided in *trans* to the Δ*crcZ*Δ*crcY* strain could partially complement the hyperrepressing phenotype of this strain, they were not high enough to reduce the repressing effect of the Hfq/Crc proteins on *benA* expression in cells cultured in complete medium, a condition in which the Hfq/Crc-dependent CCR is very strong. In fact, our results show that under the conditions analyzed, CrcZ* is present at low and constant levels, produced from the weak and constitutive *PcbrB* promoter. On the contrary, the activity of promoter *PcrcZ* varies over an order of magnitude, being highest when cells use a poor carbon source such as oxaloacetate, and lowest when cells grow in a complete medium such as LB (Valentini et al. 2014). Even in LB medium promoter, *PcrcZ* shows some activity and the primary CrcZ is present, albeit its amounts are much lower than when *PcrcZ* is fully active (Moreno et al. 2012). The fact that processing of the *cbrB-crcZ* RNA generates a sRNA that is almost identical to CrcZ, and the observation that the processed sRNA is functional, suggest that it may play some role in helping CrcZ to antagonize Hfq/CrcZ activity. Since lack of all forms of CrcZ/CrcY is very detrimental to the cells, we propose that the processed CrcZ* sRNA might help in attaining basal levels of CrcZ/CrcZ* that are sufficient to protect the cell from an excessive Hfq/Crc-dependent repression. In addition, CrcZ* may also help to increase the total output of CrcZ, making the CrcZ-dependent response faster.

The considerable sequence similarity of CrcZ and CrcY suggests that CrcY* is also functional and could participate in controlling Hfq/Crc availability. In conditions that do not require CCR, the amounts of primary CrcZ and CrcY can increase rapidly by activation of promoters *PcrcZ* and *PcrcY* via the CbrB activator, thereby sequestering the Hfq/Crc proteins.

CrcZ and CrcY amounts are very low in Crc-null (García-Mauriño et al. 2013) and Hfq-null strains (this study). The use of transcriptional fusions to *lacZ* showed that Crc and Hfq have indirect influence on *PcrcZ* and *PcrcY* promoter activity, an effect that could derive from an as yet uncharacterized Hfq/Crc-regulated protein that represses transcription from these promoters (García-Mauriño et al. 2013). Our results nonetheless show that Crc and Hfq increase CrcZ stability, which adds an additional layer of control that might help to explain why inactivating the *crc* or *hfq* genes greatly reduces CrcZ and CrcY levels. The observation that Crc and Hfq increase CrcZ stability supports previous proposals that these proteins form a complex with CrcZ (Sonnleitner and Blasi 2014; Madhushani et al. 2015; Moreno et al. 2015). This complex might not only control Hfq/Crc availability, but could also protect CrcZ from degradation by RNases. Indeed, Hfq was observed to protect several RNAs from cleavage by RNaseE, consistent with the observation that Hfq can recognize targets very similar to those of RNase E (Møller et al. 2002; Folichon et al. 2003; Massé et al. 2003; Moll et al. 2003; for review, see Saramago et al. 2014). The half-life of CrcZ, CrcY, and their processed variants is central to determining the levels of these sRNAs in different growth conditions.

Combined with previous evidence, our results indicate that CrcZ abundance in *P. putida* is controlled by the complex interaction of several elements that ultimately affect transcription of *crcZ*, as well as the processing and stability of the *crcZ* transcript. Each component affects either the transcription or the stability of other components. The overall process would be similar for *crcY*. This complex autoregulation is summarized in Figure 11.

Since Hfq and Crc abundance in *P. putida* does not appear to vary greatly in distinct growth conditions (Moreno et al. 2015), one could predict that CrcZ/CrcY would compete with mRNAs bearing appropriate A-rich motives to bind available free Hfq/Crc molecules. Efficient transition from a situation in which Hfq and Crc proteins are bound to CrcZ/CrcY (no CCR control) to the opposite configuration, in which most Hfq and Crc proteins are bound to target mRNAs (strong CCR control), would be determined by the levels of CrcZ/CrcY and of target mRNAs. Hfq has high affinity for RNA and dissociates slowly, at least in vitro. To move rapidly among different RNAs, Hfq was proposed to follow an "active cycling" process, sliding from one RNA to another in a manner not limited by the slow dissociation rates, but rather driven by the concentration of free target RNAs (Wagner 2013). In cells growing in a complex medium such as LB, which is a mixture of preferred and nonpreferred carbon sources, *PcrcZ* and *PcrcY* promoter activity is low and CrcZ and CrcY levels are also comparatively low, such that Hfq/Crc can saturate target mRNAs, inhibiting their translation. The mRNAs that specify proteins needed for the induction, uptake, and assimilation of nonpreferred compounds frequently bear A-rich sites recognized by Hfq (Hernández-Arranz et al. 2013), and Hfq/Crc can therefore inhibit induction of these nonpreferred catabolic pathways. Hfq/Crc binding to these mRNAs would leave CrcZ/CrcY insufficiently protected and exposed to degradation by RNases. A decrease in the concentration of preferred compounds would lead to induction of promoters *PcrcZ* and *PcrcY* by the CbrB activator, increasing levels of CrcZ and CrcY, which also have A-rich sites. The equilibrium would switch toward a situation in which mRNAs containing A-rich sites lose Hfq/Crc, and can therefore be translated, while CrcZ/CrcY would bind to these proteins and thus be protected from RNases. The final

configuration of the regulatory system would therefore depend on the relative levels of the target mRNAs and CrcZ/CrcY. The outcome of this complex and multilayered regulation is a rapid response that leads to the hierarchical regulation by available free Hfq/Crc proteins of several uptake and assimilation systems for different carbon sources, thereby organizing the best possible configuration of metabolism, which optimizes growth speed.

MATERIALS AND METHODS

Bacterial strains, culture media, and plasmids

E. coli and *P. putida* strains were cultured at 37°C and 30°C, respectively. Lysogeny broth (LB; 10 g/L tryptone; 5 g/L yeast extract, 10 g/L NaCl) was used as complete growth medium. M9 minimal salts medium (Sambrook and Russell 2001) was supplemented with trace elements (Bauchop and Eldsen 1960), and either 30 mM succinate, 30 mM citrate, 30 mM glucose, or 10 mM benzoate as the carbon source. When needed, antibiotics were added at the following concentrations: kanamycin (50 µg/mL), gentamicin (40 µg/mL), streptomycin (50 µg/mL, or 800 µg/mL when the host strain harbored a gentamicin-resistance determinant), ampicillin (100 µg/mL). Cell growth was followed by measuring turbidity at 600 nm.

The *P. putida* strains used were KT2440 (wild type), KT2442 (a spontaneous rifampicin-resistant derivative of strain KT2440 (Franklin et al. 1981), MPO401 (KT2442 *cbrB::km*; García-Mauriño et al. 2013), KT2442*rpoN::Ωkm* (Köhler et al. 1989), KT2440Δ*hfq* (Arce-Rodriguez et al. 2015), KTCRC (KT2440 with a *crc::tet* allele; Hernández-Arranz et al. 2013), KT2440-Z (derives from KT2440 by replacing *crcZ* with an inactivated *crcZ::tet* allele; Moreno et al. 2012), KT2440-ZY (derived from KT2440 and lacks CrcZ and CrcY sRNAs; La Rosa et al. 2015), PBA1 (derived from KT2442; bears a *PbenA::lacZ* transcriptional fusion in the chromosome; Moreno et al. 2012), PBA1C (PBA1 with an inactivated *crc::tet* allele; Moreno et al. 2012), and PBAZY (PBA1 Δ*crcZ*Δ*crcY*; Moreno et al. 2012).

To search for promoters located upstream of promoter *PcrcZ*, a 1.7 kb DNA segment containing promoter *PcbrB*, the complete *cbrB* gene, and downstream sequences to nucleotide 118 downstream from the *cbrB* stop codon (excluding *PcrcZ* sequences and the CbrB binding site upstream of it), were PCR-amplified using oligonucleotides Fw1 and RV7 (Supplemental Table S1). This DNA fragment was cloned between the EcoRI and XbaI sites of plasmid pMP220 to generate a *PcrcB-cbrB-lacZ* transcriptional fusion; the plasmid obtained was termed pFW1. Similar plasmids were constructed in which this region was progressively deleted from the 5′-end, using oligonucleotides FW2 to FW7 as direct primers for the PCR reaction (Supplemental Table S1) and RV7 as the reverse primer; the constructs were termed pFW2, pFW3, pFW4, pFW5, pFW6, and pFW7.

To generate plasmid p421-Pwt, a DNA region that includes the *cbrB* and *crcZ* genes (including the *PcbrB* promoter) was PCR-amplified from KT2440 chromosomal DNA using the Pfu high-fidelity DNA polymerase (Promega) and oligonucleotides PcbrBEcoFW1 and CrcZ-XbaI-rv (Supplemental Table S1). The DNA fragment obtained was treated with EcoRI and XbaI and cloned between the EcoRI and XbaI restriction sites of plasmid pSEVA 421 (Martínez-García et al. 2015). Plasmid p421-Pmut is a variant of p421-Pwt in which the GG and GC nucleotides at the consensus −24 and −12 regions of promoter *PcrcZ* were modified to CC and TT, respectively. To construct it, the appropriate DNA segment was chemically synthesized by GeneArt (Thermo Fisher Scientific) and cloned between the EcoRI and XbaI restriction sites of plasmid pSEVA 421.

Plasmid pPcrcZ (Fonseca et al. 2013), which derives from plasmid pMP220 (Spaink et al. 1987), contains a *PcrcZ-lacZ* transcriptional fusion. Plasmid pPcrcZmut is equivalent to pPcrcZ except that the GG and GC nucleotides at the consensus −24 and −12 regions of promoter *PcrcZ* were modified to CC and TT, respectively. To obtain this plasmid, a DNA segment spanning from the end of *cbrB* to the start site of promoter *PcrcZ* was PCR-amplified from plasmid p421-Pmut with oligonucleotides PcrcZ-EcoRI and PcrcZ-rv-BamHI-2 (See Supplemental Table S1); the amplified fragment was treated with EcoRI and BamHI and cloned between the EcoRI and BamHI sites of the reporter plasmid pMP220, to generate a transcriptional fusion to the *lacZ* indicator gene.

Plasmid p424-Z contains *crcZ* under the influence of the *Ptrc* promoter, and allows overproduction of CrcZ sRNA using IPTG as inducer (Moreno et al. 2012). All plasmid constructs were sequenced and the plasmids transferred to *P. putida* by electroporation.

Total RNA purification from *P. putida*

Cells were grown at 30°C in aerated flasks containing LB medium. At mid-exponential phase (A_{600} = 0.6) or at the start of the stationary phase (A_{600} = 2.2), samples were collected, harvested by centrifugation, and frozen at −70°C. RNA was purified from cell pellets with the RNeasy RNA purification kit (Qiagen). Purified RNA was treated with RNase-free DNase I (Turbo RNA-*free*, Ambion). RNA integrity was analyzed by agarose gel electrophoresis. The absence of DNA was confirmed by real-time PCR using primers for *rpoN* or *rpoD* (Morales et al. 2006).

Real-time RT-PCR

Real-time RT-PCR assays were performed using total RNA preparations from three independent cultures (three biological replicas). RNA was reverse-transcribed into cDNA using the cDNA Archive kit (Applied Biosystems). Real-time PCR was performed essentially as described previously (Morales et al. 2006), except that the expression profile of each gene was analyzed by absolute quantitation using a standard curve. Standard curves were constructed by serial 10-fold dilutions from 10^7 to 10^3 copies of *P. putida* genomic DNA obtained using the G-NOME DNA purification kit (MP Biomedicals). The primers used are indicated in Supplemental Table S1.

Rapid amplification of cDNA ends (5′ RACE)

5′ RACE analysis was carried out using the Gene Racer Kit (Life Technologies). Briefly, total RNA obtained as described above was treated with terminator 5′-phosphate-dependent exonuclease (TEX). Reactions were terminated by extraction with phenol and chloroform, followed by ethanol precipitation. RNA samples were resuspended in water and treated with tobacco acid pyrophosphatase (TAP). The GeneRacer RNA oligo was ligated to the RNA 5′ end with T4 RNA ligase and samples were transformed to cDNA using random hexamers and SuperScript III reverse transcriptase (Life

Technologies). To amplify the 5′ RACE product, a PCR reaction was performed using the GeneRacer 5′ primer and the indicated specific reverse primer (Supplemental Table S1). PCR products were then cloned into plasmid pCR4 and transformed into One Shot TOP10 *E. coli* using a TOPO PCR Cloning kit (Life Technologies). The junction (point of ligation) between the sequence corresponding to the RNA oligonucleotide and that of the cDNA 5′-end was determined by DNA sequencing in 14 independent transformants.

Analysis of CrcZ 5´-end heterogeneity by primer extension

The oligonucleotide use as primer for the extension reaction, end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, was CrcZ-revPE (Supplemental Table S1). RNA was obtained as indicated above and, where indicated, treated with TEX enzyme, phenolized and precipitated with ethanol. Treated or untreated RNA (15 µg) were mixed with 4 pmol of the end-labeled primer and heated (80°C, 5 min). The oligonucleotide was allowed to anneal to the RNA by incubation (23°C, 5 min). Primer extension was performed with 200 U SuperScript III (Promega) at 55°C, as indicated by the supplier. The extended cDNA products were analyzed by electrophoresis on a denaturing 6% urea–polyacrylamide gel, in parallel with a DNA sequence ladder obtained by chemical sequencing (Maxam and Gilbert 1980) of a DNA fragment obtained by PCR. The gel was dried, exposed to a phosphorimager screen and visualized using the Personal Molecular Imager (Bio-Rad).

Assay for β-galactosidase

An overnight culture of the strain of interest was diluted to a final turbidity (A_{600}) of 0.05 in fresh LB medium. Where indicated, 1 mM IPTG or 5 mM benzoate were added to induce transcription from promoters *Ptrc* or *PbenA*, respectively. Cells were allowed to grow at 30°C with vigorous aeration; aliquots were taken at various time points; and β-galactosidase activity was measured using as substrate *o*-nitrophenyl-β-D-galactoside (Miller 1972). Three independent assays were performed.

Northern blots

To generate an RNA probe against CrcZ, a 150-bp DNA segment spanning the central region of CrcZ (the least similar to CrcY) was PCR amplified using primers CrcZ-HindIII-fw and CrcZ EcoRI-rv (see Supplemental Table S1), which include restriction sites for endonucleases HindIII and EcoRI, respectively. The resulting DNA fragment was cloned between the HindIII and EcoRI sites of plasmid pSPT18 (Roche) to obtain pSPT18-Z. The plasmid was HindIII linearized and used as template for an in vitro transcription reaction in which the central region of *crcZ* was transcribed from the T7 promoter of the vector. Transcription was performed using the DIG RNA labelling kit (Roche) and T7 RNA polymerase. To generate a probe against the 5S ribosomal RNA, a DNA fragment corresponding to the gene specifying the 5S RNA was PCR amplified using primers 5S-HindIII-fw and 5S-EcoRI-rv (Supplemental Table S1), and the resulting DNA fragment was cloned between the HindIII and EcoRI sites of plasmid pSPT18 to generate plasmid pSPT18-5S. This plasmid was linearized with HindIII and used as template for an in vitro transcription reaction with T7 RNA polymerase. as indicated above.

For Northern blots, 5 µg total RNA purified from the indicated strain, and 60 ng of RNA Molecular Weight Marker III DIG-labelled (Roche), were resolved by electrophoresis on a 6% polyacrylamide gel containing 7 M urea and transferred to a Nylon Hybond N+ membrane (GE Healthcare Biosciences) with a semi-dry transfer unit (Trans-blot SD. Bio-Rad; 400 mA, 1 h). Membranes were UV-cross-linked and hybridized with a mixture of DIG-labeled probes against CrcZ and 5S (20 ng each). Bands that hybridized to the probes were detected using the DIG Luminescent Detection Kit (Roche), and signals were visualized by exposure to Agfa X-ray film. A digoxigenin-labeled RNA molecular weight marker (Roche) was used as a size marker.

Where indicated, the RNA was treated with TEX (terminator 5′-phosphate-dependent exonuclease, Epicentre) prior to Northern analyses. Briefly, 5 µg total RNA were incubated with 1 U of TEX (1 h, 30°C) and the reaction terminated by adding 1 µL of 100 mM EDTA (pH 8). Formamide buffer was added and the reaction was loaded into the 6% polyacrylamide 7 M urea gel.

Determination of CrcZ sRNA stability

P. putida strains KT2440-Z, KTCRC, and KT2440Δ*hfq* containing plasmid p424-Z were cultured at 30°C in LB medium supplemented with streptomycin. To induce CrcZ synthesis, IPTG was added to a final concentration of 1 mM. When cultures reached a turbidity of 0.6 (A_{600}), 0.3 mg/mL rifampicin was added; 10 mL aliquots were withdrawn at 0, 2, 4, 8, 16, and 32 min and mixed with two volumes of RNAprotect Reagent (Qiagen). Total RNA was isolated as above and 10 µg of the RNA obtained were resolved on a 6% polyacrylamide/7 M urea gel. The RNA bands were transferred to a nylon Hybond N+ membrane and detected by Northern blot as above. Band intensities were quantified using a ChemiDoc XRS imager and *Image Lab* software (Bio-Rad). The value obtained for CrcZ in each sample was normalized to that of the 5S rRNA of the same sample.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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Effect of Crc and Hfq proteins on the transcription, processing, and stability of the *Pseudomonas putida* CrcZ sRNA

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