

The Crc global regulator binds to an unpaired A-rich motif at the *Pseudomonas putida alkS* mRNA coding sequence and inhibits translation initiation

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Received September 3, 2009; Revised and Accepted September 18, 2009

ABSTRACT

Crc is a key global translational regulator in Pseudomonads that orchestrates the hierarchy of induction of several catabolic pathways for amino acids, sugars, hydrocarbons or aromatic compounds. In the presence of amino acids, which are preferred carbon sources, Crc inhibits translation of the *Pseudomonas putida alkS* and *benR* mRNAs, which code for transcriptional regulators of genes required to assimilate alkanes (hydrocarbons) and benzoate (an aromatic compound), respectively. Crc binds to the 5'-end of these mRNAs, but the sequence and/or structure recognized, and the way in which it inhibits translation, were unknown. We have determined the secondary structure of the *alkS* mRNA 5'-end through its sensitivity to several ribonucleases and chemical reagents. Footprinting and band-shift assays using variant *alkS* mRNAs have shown that Crc specifically binds to a short unpaired A-rich sequence located adjacent to the *alkS* AUG start codon. This interaction is stable enough to prevent formation of the translational initiation complex. A similar Crc-binding site was localized at *benR* mRNA, upstream of the Shine–Dalgarno sequence. This allowed predicting binding sites at other Crc-regulated genes, deriving a consensus sequence that will help to validate new Crc targets and to discriminate between direct and indirect effects of this regulator.

INTRODUCTION

Free-living bacteria, particularly those species containing large genomes, usually have a versatile metabolism that allows the use of many different compounds as a source

of carbon and energy. This facilitates survival in different habitats and adaptation to changing environmental conditions. Metabolic versatility is linked to a tight though flexible regulation of the expression of the different metabolic pathways involved, which occurs through both specific and global regulation systems. Several distinct global regulation networks can participate in coordinating gene expression programmes under different situations (1). Some of them allow cells to respond to stress conditions such as starvation or oxygen limitation. Many bacteria possess as well global regulation systems that coordinate metabolism under conditions of feast, i.e. when several possible carbon sources are available at high concentrations. The regulatory outcome, named catabolite repression control, allows cells to induce the genes required for the assimilation of a preferred compound, inhibiting at the same time the uptake and/or the expression of genes required for the catabolism of other non-preferred compounds, adapting metabolism accordingly. Catabolite repression can have an important impact on cell behaviour, modifying for example the expression of virulence factors and the infecting ability in pathogenic bacteria (2).

The molecular mechanisms responsible for catabolite repression control can differ markedly in distinct bacterial species (2,3). In Pseudomonads, which are characterized by a great metabolic flexibility, catabolite repression is still poorly understood, in spite of the importance of these bacteria in biotechnology, in medicine and in the environment. Available evidence indicates that, in these bacterial species, catabolite repression occurs through mechanisms that are very different from those present in other model organisms such as *Escherichia coli* or *Bacillus subtilis* (4). Several global regulatory mechanisms leading to catabolite repression control have been identified in *Pseudomonas aeruginosa* and in *P. putida*. One of these systems relies on the Crc protein, a master regulator of carbon metabolism. Crc represses the expression of genes involved in the assimilation of some sugars, nitrogenated

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compounds, aromatic compounds and hydrocarbons when other preferred substrates are present (5–12). Contrary to what has been observed in other bacterial species, succinate or some amino acids are preferred over glucose in Pseudomonads. A recent genomic and proteomic analysis has shown that Crc coordinates the hierarchical use of amino acids in *P. putida* when cells grow in a complete medium, affecting the expression of more than 130 genes (13). Most of these effects are probably indirect. The molecular mechanism through which Crc inhibits gene expression has been determined for the alkane and for the benzoate degradation pathways (14,15). In both cases, Crc directly controls the expression of the transcriptional activator of the pathway genes (AlkS for the alkane pathway, and BenR for the benzoate pathway). Crc is an RNA-binding protein that binds to the 5'-end of the *alkS* and *benR* mRNAs, inhibiting their translation *in vivo* (14,15). Thus, by decreasing the synthesis of AlkS, or of BenR, Crc indirectly reduces the mRNA levels of all the genes that are under the influence of these two specific transcriptional activators. Crc does not contain a known specific RNA-binding motif. Moreover, no information exists on the RNA determinants that are specifically recognized by Crc.

In this work, we report the characterization of the Crc-binding site at the *alkS* mRNA by using a combination of footprinting techniques and mutagenesis analysis. Our data show that Crc binds to an unpaired AU-rich sequence motif in the coding sequence of *alkS* mRNA and prevents the formation of active ribosomal initiation complex. Crc was found to bind to a similar AU-rich motif at the *benR* mRNA. Based on these two targets, other potential Crc-binding sites were predicted for several Crc-repressed genes. These data will help to discriminate between direct and indirect effects of this global regulator, in order to unravel the Crc-dependent regulatory network.

MATERIALS AND METHODS

Protein purification

The Crc protein containing a 6xHis tag at the C-terminus, which is biologically active, was overproduced in *E. coli* BL21(DE3) (pLysS) containing plasmid pCRCH, and purified using an Ni-NTA column, as described previously (14). *E. coli* BL21(DE3) (pLysS) (16) and plasmid pCRCH, which contains the *crc(6xHis)* gene (14), have been described earlier.

RNA fragments for *in vitro* analyses

The 26 or 33 nt RNA oligonucleotides used were chemically synthesized and obtained from Sigma. Larger RNA fragments were obtained by *in vitro* transcription with T7 RNA polymerase, using as substrate *PstI*-linearized plasmids palkS-78 or palkS-200. These plasmids, which derive from pGEM-T-Easy (Promega), contain different segments of the 5' leader of *alkS* mRNA (positions 1–78 or –37 to 200, respectively, relative to promoter *PalkS2* transcription start site) cloned between the *ApaI* and *PstI*

sites of the vector. These *alkS* segments can be transcribed from the T7 promoter of the vector. The run-off transcripts obtained contain, in addition to the mentioned *alkS* mRNA segments, 14 extra nts at the 5'-end and one extra nt at the 3'-end, derived from vector sequences. Transcription was performed for 1 h at 37°C; reactions (1 ml) contained 40 mM Tris-HCl (pH 8), 15 mM MgCl₂, 50 mM NaCl, 5 mM spermidine, 5 mM DTT, 4 mM ATP, CTP GTP and UTP, bovine serum albumin at 50 µg/ml, RNasin (300 U), 0.1 µg/ml of DNA template (linearized with *PstI*), and 800 U of T7 RNA polymerase. The RNA transcript was phenol-extracted and purified on a denaturing 8 M urea–8% polyacrylamide gel. The full-length RNA transcript corresponding to the *alkS* leader region was eluted in a buffer containing 0.5 M ammonium acetate, 1 mM EDTA, 16% phenol and 0.1% SDS overnight at 4°C, phenol-extracted and precipitated with ethanol. Gel-purified RNA was dephosphorylated with calf intestine alkaline phosphatase (Roche).

The 5'-end labelling of RNA fragments, RNA oligonucleotides, or DNA oligonucleotides was performed with T4 polynucleotide kinase and [γ -³²P]ATP, as described (17). Labelled RNAs were purified on a denaturing 8% polyacrylamide–urea gel, eluted and precipitated with ethanol. Small RNA oligonucleotides were purified through MicroSpin G-25 columns (GE Healthcare).

Probing of mRNA secondary structure

RNA secondary structure probing was performed essentially as described (18). Prior to its use, 5'-end-labelled RNA was denatured for 1 min at 90°C, chilled on ice for 3 min, and allowed to fold at 20°C in 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 50 mM KCl. One microgram of tRNA was then added and RNA was digested with RNase T1 (0.5 and 1 U), RNase T2 (0.05 and 0.1 U), RNase V1 (0.005 and 0.0025 U) or RNase A (0.0025–0.01 µg/ml) for 6 min at 20°C. RNases were added at concentrations optimized to yield no more than one cut per RNA molecule. Reactions were stopped by adding 20 µl of stop solution (1 M guanidine thiocyanate, 0.167% *N*-lauryl-sarcosine, 10 mM DTT, 83% isopropanol) and precipitated with ethanol. Digestion products were resolved on denaturing 8% urea–polyacrylamide gels.

Footprinting assays

Footprinting was performed with either unlabelled RNA, or with 5'-end-labelled RNA, prepared as described above. The RNA, previously renatured, was incubated for 15 min at 20°C in 10 µl reactions containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 50 mM KCl, 1 µg of total tRNA, in the absence or in the presence of purified Crc protein (3 nM to 2 µM). The free mRNA and the protein-mRNA complexes were then probed for 6 min at 20°C with RNase T1, RNase T2, or RNase V1 as described above. For dimethylsulfate (DMS) modification, 1 µl DMS (freshly diluted 1:16 in ethanol) was added to 25 µl of 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 50 mM KCl, 1 µg of total tRNA, *alkS* mRNA (2 pmol) and, when added, Crc (3 nM to 2 µM).

Reactions were allowed for 5 min at 20°C. Analysis of the sensitivity of riboses towards modification by the 1M7 reagent (SHAPE assays) was also performed using non-labelled *alkS* mRNA, alone or bound to Crc as described above, in the presence of 2 and 4 mM of 1M7 reagent dissolved extemporaneously in DMSO. The reaction was performed for 70 s at 20°C following the conditions described earlier (19). In all cases, reactions were stopped by phenol–chloroform extraction, followed by ethanol precipitation.

When an end-labelled RNA was used, the digestion products were analyzed by electrophoresis on denaturing 8% polyacrylamide–urea gels. For enzymatic and chemical reactions performed with non-labelled RNAs, the sites of digestion, and of DMS and 1M7 modifications, were revealed by primer extension using AMV reverse transcriptase, as previously described (18), and an end-labelled oligonucleotide (5'-gcaactatgaactttgge-3') which is complementary to nts +120 to +103 of *alkS* mRNA. Incubation controls (in the absence of the RNases, DMS, or 1M7) were also performed in parallel to detect unspecific nicks in the RNA or pauses of reverse transcriptase. To assign the cleavages or modifications, sequencing ladders were run in parallel.

Quantification of the reactivity of the 2'-OH group of riboses towards 1M7 was performed with the SAFA software after scanning the autoradiographies (20). The global intensity of each lane was first normalized according to the intensities of the full-length extension product bands. The intensity of each band was determined by using a scale in which the value 0 indicates an unreactive site and the value 1.0 corresponds to the average intensity at highly reactive sites (21). The normalization factor for each dataset was determined after exclusion of the most-reactive peak intensities (2%) and the average was thus calculated for the next 8% of peak intensities. All reactivities were then divided by this average. The reactivity towards 1M7 of riboses in free RNA was quantified by subtracting the unspecific pauses observed in the incubation control lane of unmodified RNA, while the reactivity of riboses of the RNA complexed with Crc was subtracted from the densities found in the control of unmodified RNA bound to Crc. Reactivity of each ribose was classified as high, medium or low. Comparison of ribose reactivity on the RNA in the absence or presence of Crc reveals the changes induced by Crc binding.

RNA band shift assays

Radioactively labelled RNA (0.1 nM) was incubated for 30 min at 20°C, in the absence or presence of purified Crc (53–1700 nM), in 20 µl reactions containing 10 mM HEPES–KOH (pH 7.9), 35 mM KCl, 2 mM MgCl₂ and 1 µg of yeast tRNA. When indicated, competing unlabelled RNA oligonucleotides were also added. After adding 4 µl of loading buffer (60% glycerol, 0.025% xylene cyanol), complexes were resolved by electrophoresis under non-denaturing conditions using 4 or 6% polyacrylamide–urea gels, as described (14).

Determination of the *benR* transcription start site

Cells of *P. putida* KT2440 (22) were collected by centrifugation, and total RNA was obtained using the RNeasy Mini kit (Qiagen). Thirty micrograms of RNA were denatured by heating to 85°C for 3 min, and annealed at 20°C with 2.5 pmol of the oligonucleotide 5'-gaaacacgctactgcgctcg-3', which was end labelled at the 5'-end with [γ -³²P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase. The oligonucleotide is complementary to nts 40 to 21 relative to the *benR* translational start site. The RNA was precipitated with ethanol and resuspended in 10 µl of water. Primer extension reactions, performed essentially as described (23), were conducted for 1 h at 42°C using 10 U of AMV reverse transcriptase. The extended cDNA products were analyzed by electrophoresis on 6% polyacrylamide–urea gels. A DNA sequence ladder was performed by chemical sequencing of a DNA fragment of the *benR* promoter region, obtained by PCR with oligonucleotides 5'-gaaacacgctactgcgctcg-3' (labelled at the 5'-end) and 5'-gaccgtgatgaccgcaacca-3'. The same labelled oligonucleotide was used for primer extension and the sequence reactions allowing the precise identification of the 5' start of *benR* mRNA.

Extension inhibition (Toeprint) assays

Formation of simplified translational initiation complexes was performed using *E. coli* 30S ribosome subunits, essentially as described (24,25). The *alkS* mRNA was obtained by *in vitro* transcription with plasmid palkS-200 (see 'RNA fragments for *in vitro* analyses' section). Reactions contained, in 10 µl, 25 nM of renatured mRNA, the 5'-end-labelled primer (complementary to nts +103 to +120 relative to *PalkS2* transcription start site), which was pre-annealed to the mRNA, 200 nM 30S subunits, 20 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 60 mM KCl, 1 mM DTT and, when indicated, Crc protein at various concentrations (16 nM to 2 µM). After 15 min at 37°C, all four dNTPs (each up to 50 µM) and tRNA_f^{Met} (2 µM) were added and the assays were incubated for a further 5 min at 37°C. Primer extension was conducted with 1 U of AMV reverse transcriptase for 15 min at 37°C. Reactions were stopped by phenol–chloroform extraction, followed by ethanol precipitation. The cDNA formed was fractionated on denaturing 8% polyacrylamide–urea gels.

RESULTS

The secondary structure of the *alkS* mRNA leader sequence

The *alkS* gene can be expressed from two promoters (Figure 1). In the absence of alkanes, *alkS* is transcribed from promoter *PalkS1* (26). The AlkS protein binds to this promoter, acting as a repressor and allowing for a low basal expression. When alkanes become available, AlkS turns into a transcriptional activator and, from its site at *PalkS1*, it activates the expression of promoter *PalkS2*, located 38 bp downstream from *PalkS1* (27).

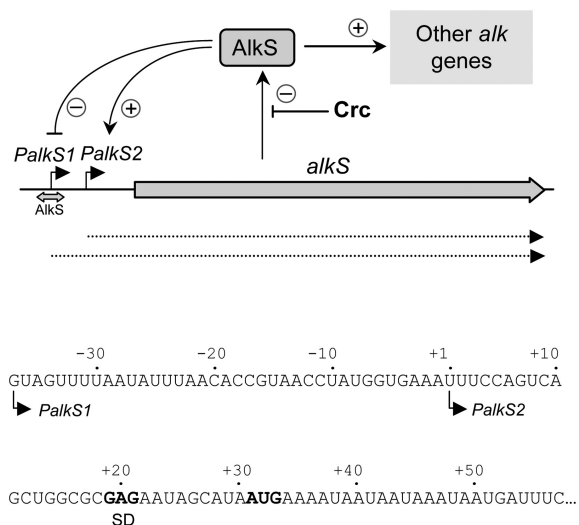


Figure 1. Transcription of the *alkS* gene. The *alkS* gene can be transcribed from two promoters, *PalkS1* and *PalkS2*. In the absence of alkanes, *alkS* is expressed from promoter *PalkS1*, which is recognized by σ^S -RNA polymerase. AlkS binds to a site overlapping the *PalkS1* -10 region, repressing this promoter but allowing for a low basal expression. When alkanes become available, AlkS activates the expression of promoter *PalkS2*, located 38 bp downstream from *PalkS1*. This leads to a self-amplification process that significantly increases *alkS* mRNA levels. AlkS also activates the expression of other genes of the alkane degradation pathway from promoter *PalkB* (data not shown). Under conditions generating catabolite repression control (i.e. in the presence of amino acids that are preferred to alkanes), translation of the *alkS* mRNA is inhibited by the Crc global regulatory protein. The sequence of the *alkS* mRNA is shown (the start sites corresponding to promoters *PalkS1* or *PalkS2* are indicated; coordinates are referred to the *PalkS2* start site). The Shine-Dalgarno (SD) sequence and the initiation codon AUG are indicated in bold characters.

We have previously shown that Crc can efficiently bind *in vitro* to an RNA fragment that includes the first 43 nt of the *alkS* mRNA that starts at promoter *PalkS2* (14). Binding is also efficient when the 3'-end of the mRNA extends up to position +99. The Crc-RNA interaction is specific, since it can be competed by an excess of specific RNA, but not by a similar amount of a non-specific RNA (14).

Prior to characterizing the Crc-binding site, the secondary structure of the *alkS* mRNA 5'-end was analyzed using the double-strand-specific RNase V1 and several single-strand-specific RNases (RNases T1, T2, A). Results were interpreted with the help of computer simulations of RNA folding at <http://mfold.bioinfo.rpi.edu> (28). The RNA molecule used, which includes nts +1 to +78 of *alkS* mRNA (abbreviated as 1-78 *alkS* RNA; coordinates are referred always relative to promoter *PalkS2* transcription start site), was labelled at the 5'-end and renatured in the presence of magnesium ions. The secondary structure model derived from the enzymatic cleavage patterns show two stem-loop structures (Figure 2). The largest one includes the AUG start site and a 14 nt A-rich apical loop spanning positions +33 to +46. Most of the residues located in unpaired regions were strongly cleaved by RNases A and T2. Conversely, the helical regions interrupted by an internal

loop were supported by the existence of several RNase V1 cleavages (at +18, +20, +29, +51 and +59). However, the helix that closes the apical loop is of weak stability, as deduced from the simultaneous presence of RNase V1 and RNase T2 cleavages (Figure 2B).

The experiments were also performed with a longer fragment of *alkS* mRNA that includes the *PalkS1* start site, and thus carries 37 additional nucleotides at the 5'-end. Assays were performed on the non-labelled RNA and the modifications revealed by primer extension with AMV reverse transcriptase. The accessibility of unpaired regions of the RNA were analyzed using RNases A and T2 (Figures 2A and 3A). In addition, we probed the reactivity of adenines at N1 and cytosines at N3 using DMS (Figure 3A, summarized in Figure 3D), as well as the reactivity of the 2'-OH group of riboses using 1M7 reagent (Figure 3B and C). This chemical gives insight on the local nucleotide flexibility, since a nucleotide involved in Watson-Crick base pairing or in tertiary interactions does not react with 1M7, while flexible regions (unpaired nucleotides, or a helix of weak stability) are sensitive to 1M7. This compound has several advantages: it reacts in the same manner with the four nucleotides, the reaction is very fast (below 1 min), and undergoes a self-inactivating hydrolysis (19,29).

The nucleotides showing strong or medium reactivity towards DMS (Figure 3A and D) were all located at regions that were also sensitive to RNases T2 and A (Figure 2B), thus confirming that these positions are mostly in an unpaired configuration. Modification of riboses by 1M7 gave correlated changes showing a high degree of flexibility in the regions 33-47, flanked by regions of restricted flexibility (see Figure 3B and C). The weak reactivity of riboses in the stems of this hairpin towards 1M7, and of several N1 positions of adenines towards DMS (Figure 3), strongly suggests that the hairpin including the AUG start codon is of weak stability. These results also show that the 37 additional residues in the 5'-end of the mRNA do not affect the formation of this hairpin.

Crc protects the AU-rich apical loop at hairpin II of *alkS* mRNA

To identify the Crc-binding site at *alkS* mRNA, the 5'-end-labelled 1-78 *alkS* RNA fragment was incubated in the presence of increasing concentrations of purified Crc protein prior to addition of the probe. A clear footprint was detected with RNase T2 (Figure 2), which cuts preferentially at unpaired A residues. The main protections were observed from positions 30-45. Conversely, no Crc footprint was detected with RNase V1 (Figure 2A), or with RNase T1, which cleaves unpaired guanines (not shown).

The Crc-RNA complex was further characterized by monitoring the modifications of bases and riboses with DMS and 1M7, respectively. Due to their small sizes, these two reagents are less sensitive to steric hindrance than the bulky RNases. These experiments were performed on the non-labelled (-37/+200) *alkS* mRNA, which includes *PalkS1* start site, and the

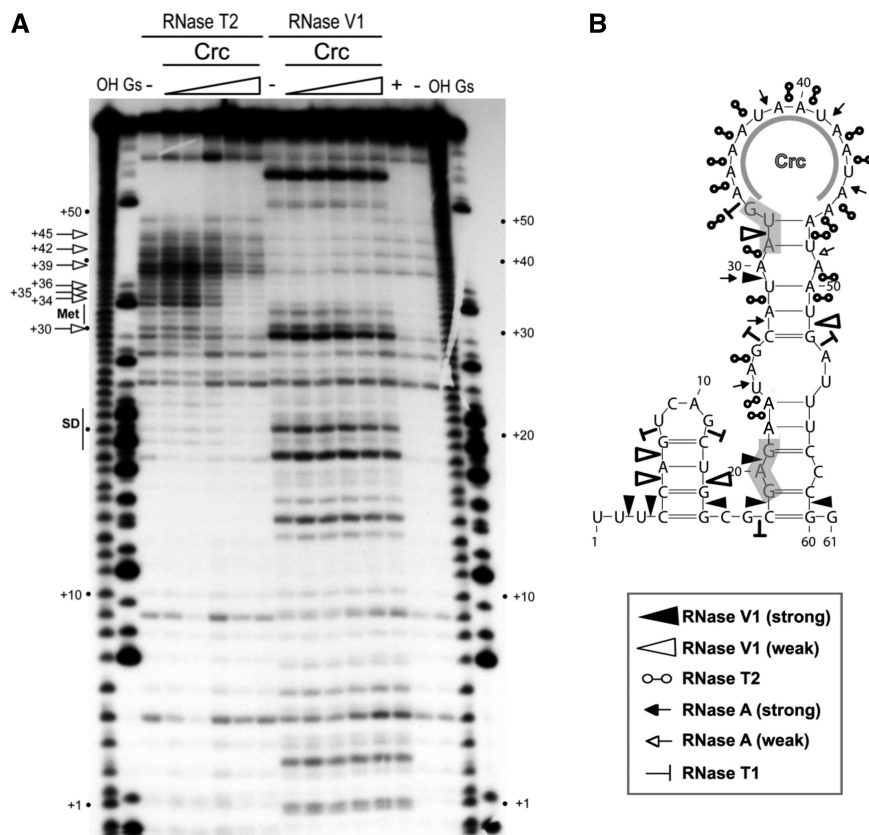


Figure 2. Secondary structure of the *alkS* mRNA leader region and effect of Crc binding monitored by enzymatic probing. (A) Fractionation of a 5'-end-labelled *alkS* mRNA fragment (nts +1 to +78) digested with RNase T2 and RNase V1 on a 12% polyacrylamide-urea gel. The enzymatic digestions were performed in the absence (–) or in the presence of increasing concentrations of Crc (3, 16, 80, 400 nM or 2 μM). Incubation controls (in the absence of RNase) were done in the absence (–) and in the presence (+) of Crc (80 nM). Lanes 'OH' and 'Gs' correspond, respectively, to an alkaline ladder and an RNase T1 ladder performed under denaturing conditions. Arrows indicated the nucleotides protected by Crc from the attack of RNase T2. Positions relative to *PalkS2* transcription start site, as well as the Shine-Dalgarno sequence (SD) and the AUG codon (Met), are indicated on the sides of the gel. (B) Summary of the cleavage patterns generated by RNase V1, RNase T2, RNase A and RNase T1 depicted on the secondary structure model of the *alkS* mRNA. The symbols used for each RNase are indicated in the insert. The region protected by Crc in the apical loop, as deduced from (A), is indicated. The SD sequence and the AUG start codon are shaded.

modifications were revealed by primer extension with AMV reverse transcriptase. Binding of Crc to *alkS* mRNA induced a strong reduction of DMS reactivity at position N1 of adenines +37, +39, +40, +42, +43 and +45 and, to a lesser extent, at +46 (Figure 3A and D). These data correlate well with those obtained with reagent 1M7, since Crc binding generated strong protections towards the attack of 1M7 at riboses +39, +40, +43, +46 and +47, and weaker protections at riboses from +33 to +36 (Figure 3B and C). In addition, all these nucleotides lie within the region protected by Crc from RNase T2 (Figure 3E).

Altogether, these results indicate that Crc interacts with the unpaired RNA region located between positions +37 and +47 (Figures 2 and 3), and that its binding does not significantly alter the overall secondary structure of the RNA.

A minimal RNA fragment containing the AU-rich sequence binds to Crc

To assess the relevance of the RNA regions located upstream and downstream of the identified Crc-binding

sequence, the ability of Crc to recognize different RNA segments of *alkS* mRNA was analyzed by band-shift assays. We have previously shown that the 3'-end of *alkS* mRNA can be deleted up to nt +43 without impairing Crc binding to the RNA, whereas further deletion up to position +33 eliminates Crc binding (14). On the basis of the footprinting data, a 26 bp RNA spanning positions +27–+52 (oligonucleotide 27–52_aS) was synthesized and labelled at the 5'-end. As shown in Figure 4, Crc could bind to this 26 nt RNA. Binding was competed by a 50-fold excess of the same non-labelled RNA, but not by a similar amount of a non-labelled RNA that lacks the sequences protected by Crc. Hence, the regions upstream of nt 27 or downstream of nt 52 are not strictly required for Crc binding.

To further characterize the Crc-binding region delimited by the footprinting assays, seven RNA oligonucleotides were synthesized with a sequence derived from that of oligo 27–52_aS (wild-type sequence), but containing mutations at several positions within those protected by Crc from the attack of DMS or of 1M7. The ability of Crc to bind to these RNAs was determined by band-shift

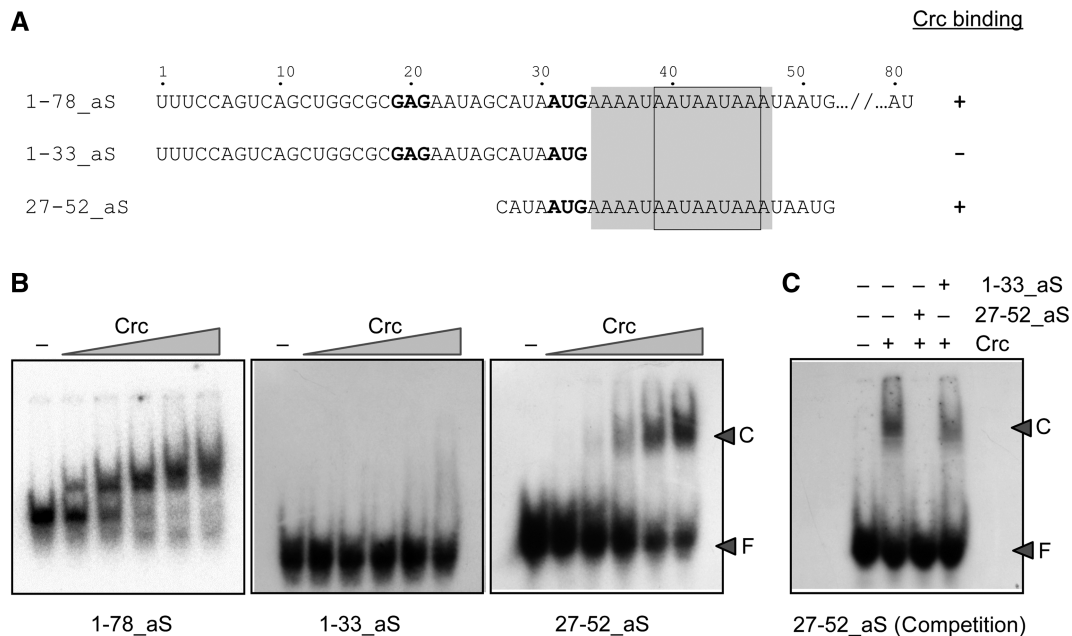


Figure 4. Determination of *alkS* mRNA minimal fragment required for Crc binding. (A) Sequence of the *alkS* mRNA fragments and ability to bind to Crc, as deduced from (B). The Crc footprint is shaded. The Crc-consensus recognition site, as defined in Figure 8, is boxed. The SD sequence and the AUG initiation codon are indicated in bold characters. (B) Band shift assays showing the ability of Crc to bind to the different *alkS* mRNA fragments. The mRNAs were incubated in the absence (-) or in the presence of increasing concentrations of Crc protein (53, 106, 212, 425 or 850 nM) in the presence of 1 μ g of tRNA. 'F' is for free RNA and 'C' is for the Crc-RNA complex. (C) Ability of the unlabelled RNA oligonucleotides 1-33_aS and 27-52_aS to compete the binding of Crc (212 nM) to the 5'-end-labelled 27-52_aS RNA. Competition was performed by adding a 50-fold excess of the non-labelled 27-52_aS RNA (specific competitor), or of the 1-33_aS RNA (non-specific competitor).

started at an A residue located 30 nt upstream from the AUG translation start site (Figure 6A). The mRNA leader region showed a sequence spanning nts +3 to +9 relative to the 5'-end that was highly similar to the central region of the Crc-binding site determined at *alkS* (AACAAUAA in *benR* as compared to AAUAAUAA in *alkS*; shaded in Figure 6B). To determine whether this sequence represents a true Crc-binding site, a 26 nt RNA spanning positions +1 to +26 of *benR* mRNA (named 1-26_bR) was synthesized and labelled at the 5'-end. Band-shift assays showed that Crc could bind to this RNA minimal site (Figure 6C, left panel). Binding was specific since it was competed when using an excess of the same non-labelled RNA, while an unlabelled RNA lacking a Crc-binding site was not able to compete (Figure 6C, central panel). Furthermore, the unlabelled 1-26_bR RNA could compete the binding of Crc to the end-labelled RNA containing positions 27-52 of the *alkS* mRNA (Figure 6C, right panel), which provides an additional evidence that the 5'-end of the *benR* mRNA contains a Crc-binding site.

The interaction of Crc with *alkS* mRNA prevents the formation of the translation initiation complex

Although it was previously shown that Crc regulates the synthesis of AlkS at a post-transcriptional level *in vivo*, it was not known which steps of translation were affected by the binding of Crc. This is of interest since Crc binds to the coding sequence of *alkS* mRNA. To investigate this issue, we analyzed the effect of Crc on the formation of the translation initiation complex at *alkS* mRNA using

toeprinting assays. This technique detects ribosomal complexes by their ability to block the extension of a cDNA from a complementary oligonucleotide primer, when the reverse transcriptase collides with the translational initiation complex (30). An *alkS* RNA fragment containing nts -37 to +200 was incubated with the *E. coli* ribosomal 30S subunit and tRNA^{Met}, in the presence or absence of Crc. The complexes formed were monitored by extension of a primer hybridizing between positions +120 and +103. As shown in Figure 7A, in the absence of Crc a clear stop of reverse transcriptase (toeprint) was detected at position +16 relative to the AUG start codon, which indicates that the translation initiation complex has formed, in spite of the weak SD sequence. No such stop was detected when either 30S ribosome or tRNA^{Met} were omitted in the reaction. The presence of increasing concentrations of Crc progressively inhibited the toeprint at position +16, which indicates that Crc binding is sufficiently stable to impair formation of the translation initiation complex. A toeprint corresponding to Crc binding was not clearly observed, suggesting that reverse transcriptase is effective at displacing Crc from its binding site.

DISCUSSION

The results presented show that the Crc global regulatory protein represses translation of the *alkS* mRNA by sequestering part of the coding sequence just downstream of the AUG start codon. Footprinting assays indicate that

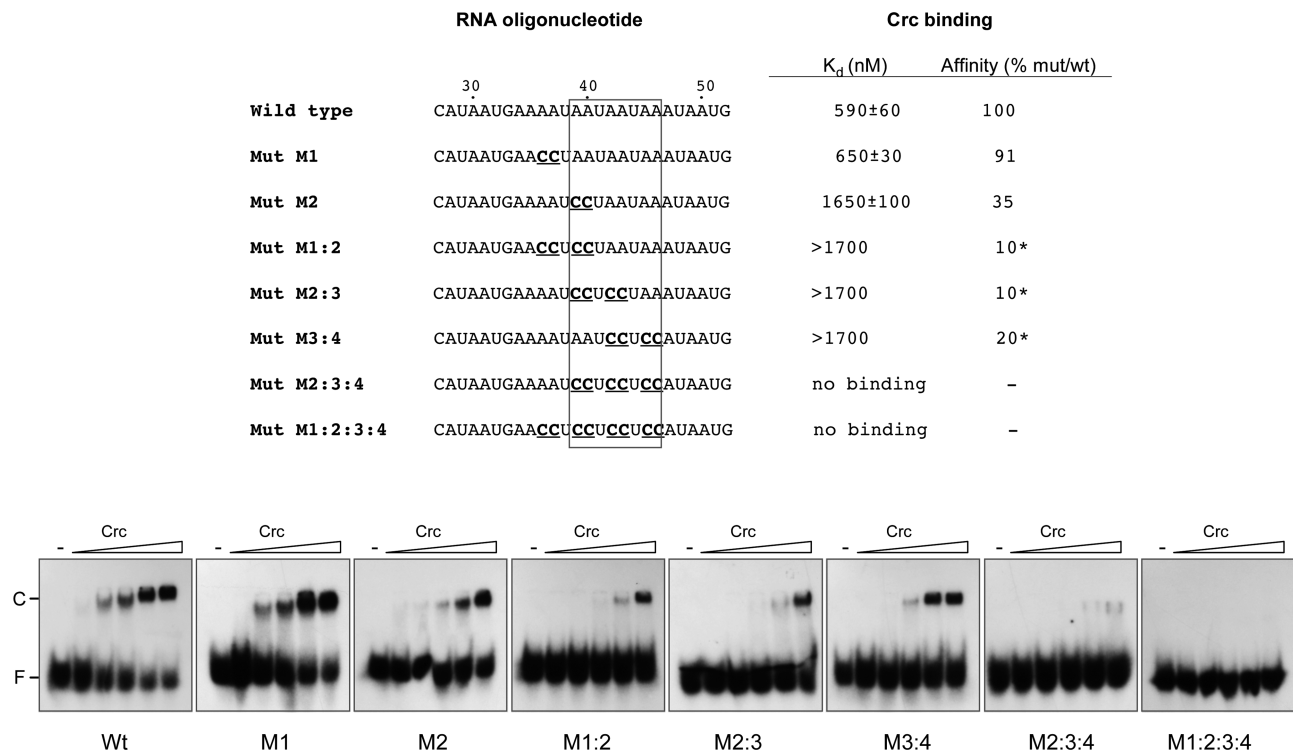


Figure 5. Mutational analysis of the Crc-binding site at the *alkS* gene. The ability of Crc (106, 212, 425, 850 or 1700 nM) to bind to the indicated radioactively labelled RNA oligonucleotides was determined by band-shift assays in the presence of 1 μ g of tRNA. The free RNA and retarded band corresponding to the Crc-RNA complex are indicated as 'F' and 'C', respectively. The wild-type RNA includes the *alkS* positions 27–52 (oligo 27–52_aS), while the mutant oligonucleotides indicated below have different substitutions within this sequence (indicated in bold and underlined). The boxed sequence corresponds to the Crc-consensus recognition site (as defined in Figure 8). The apparent binding constant (K_d) for the wild-type RNA, and for the mutant RNAs M1 and M2, was evaluated after quantification of the radioactive bands of the free and Crc-bound RNAs with a Molecular Imager and the QuantityOne software (BIORAD), and corresponds to the concentration of the protein allowing 50% binding, since the concentration of the labelled RNA was negligible. The K_d value could not be evaluated for mutant RNAs M1:2, M2:3 and M3:4 in the Crc concentration range tested. The effect of the mutations on Crc binding is indicated as percent relative to that observed for the wild-type RNA. For mutants M1:2, M2:3 and M3:4, the value was estimated by comparing the amount of Crc needed to retard 15% of the free RNA relative to that needed for the wild-type RNA (indicated with an asterisk).

Crc binds specifically to the sequence motif (+37)AUAAUAAUAAA(+47) of *alkS* mRNA. Analysis of the 2D structure of this mRNA region suggested that the Crc-binding site has an unpaired configuration, and lies within the apical loop of an unstable stem-loop structure. Mutational analysis of this RNA region supports that the sequence of this AU-rich motif is important for an efficient binding of Crc, being residues A₃₉ and A₄₀ particularly significant. The data also show that the changes induced by Crc on the RNA are restricted to the AU-rich unpaired region, since Crc binding did not alter significantly the overall secondary structure of the mRNA upstream or downstream of the binding site. This is further supported by the fact that a small RNA substrate containing the AU-rich motif (nts +27 to +52) specifically binds to Crc, albeit with a weaker affinity than for the longer RNA fragments.

Crc can also bind to the 5'-end of the *P. putida benR* mRNA, inhibiting translation (15). The results presented herein have allowed identifying a Crc-binding site at *benR* mRNA located between nucleotides +3 and +9. Computer analyses predict that this Crc-binding site lies within an unpaired RNA region. Unlike in the case of

alkS, the Crc-binding site at *benR* is located a few nt upstream of the predicted Shine-Dalgarno (SD) sequence. The similarity between the *alkS* and *benR* Crc-binding sites is restricted to the sequence AA(C/U)AAUA A (Figure 8A), which includes most of the nucleotides protected by Crc from the attack of DMS at *alkS* mRNA. This consensus motif offers the possibility to search for additional Crc targets. Using a proteomic approach, we recently identified 58 *P. putida* proteins, the levels of which increase in cells growing exponentially in LB medium upon inactivation of the *crc* gene (13). Thus, increased protein levels in the Δ *crc* mutant strain suggest that Crc could directly inhibit translation of some of the corresponding mRNAs. Several translational regulatory proteins recognize conserved sequence or structure signatures on their target mRNAs, in this way coordinating the expression of functionally related genes (31,32). Therefore, based on our study on *alkS* and *benR* mRNAs, we searched for a conserved Crc sequence motif in the translation initiation regions of the mRNAs coding for the 58 Crc-repressed proteins. This search was done within a window comprising nts -70 to +16 of the mRNAs since the translational initiation sites can be

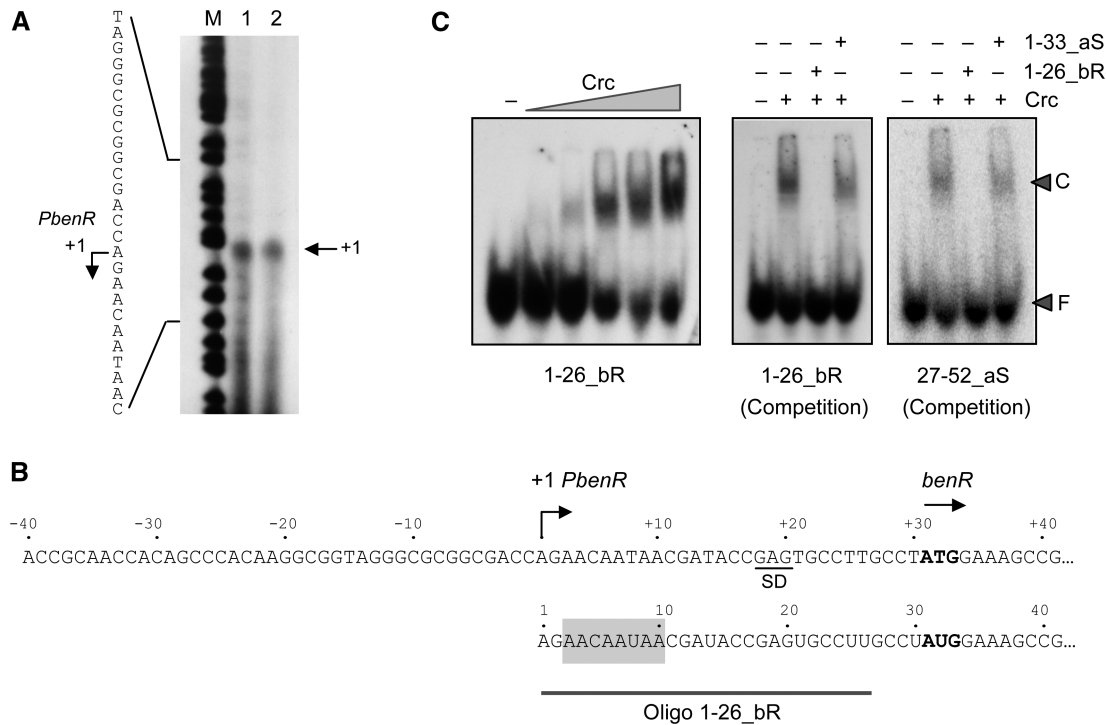


Figure 6. Identification of the Crc binding site at *benR* mRNA. **(A)** Determination of the *benR* transcription start site by primer extension. Two identical primer extension reactions were analyzed on a urea-polyacrylamide gel, in parallel to a DNA sequence ladder obtained by chemical sequencing (the bands indicate C and T residues) (43). **(B)** Sequence of the promoter for the *benR* gene, indicating the transcription start site and the AUG initiation codon (in bold); the 5'-end of the *benR* mRNA is shown below; the sequence showing similarity to Crc binding site at *alkS* mRNA is shaded, and the RNA used in panel C is indicated. **(C)** Binding of purified Crc protein to 26 nt end-labelled RNAs spanning positions from +1 to +26 of *benR* mRNA (oligo 1-26_bR), or from +27 to +52 of *alkS* mRNA (27-52_aS). Left panel: the end-labelled 1-26_bR (0.1 nM) was incubated with 1 µg of tRNA as non-specific competitor, and in the absence (-) or in the presence of increasing concentrations of Crc (53, 106, 212, 425 or 850 nM). Competition experiments contained end-labelled oligonucleotide 1-26_bR (central panel), or end-labelled oligonucleotide 27-52_aS (right panel), Crc (212 nM), 1 µg of tRNA and, when indicated, a 50-fold excess of the unlabelled RNA oligonucleotides 1-26_bR (specific competitor) or 1-33_aS (non-specific competitor, corresponding to nts +1 to +33 of *alkS* mRNA).

quite extended (31,33). Interestingly, we found 20 mRNAs that contained a sequence resembling the *alkS* and *benR* Crc-binding sites close to the ribosome binding site (Figure 8B). Eight of the genes contain the consensus sequence AA(C/U)AA(C/U)AA, while the other ones show a degenerated sequence rich in A residues. It is worth noting that, in many cases, the presumed Crc-binding site was present at the first gene of an operon (*oprB-1*, *bkdA1*, *phhA*, *hmgA*, *dadA-2* or *aspA*). One of the mRNA targets encodes the transcriptional regulator FruR, which regulates genes for the assimilation of fructose. The functional significance of this consensus and the mechanism by which Crc might repress these genes remain to be experimentally determined. Furthermore, the remaining 38 proteins detected in the proteomic assays as being repressed by Crc, but which do not show a putative Crc-binding site in their mRNA, are probably regulated by Crc in an indirect way. Taken together, our prediction will likely be helpful to validate new direct Crc targets and to discriminate between direct and indirect effects of this global regulatory protein.

The results presented also indicate that Crc impedes the formation of the 30S-tRNA^{Met}-RNA ternary complex at the *alkS* mRNA. Inhibition of translation initiation by a *trans*-acting repressor can occur through different

mechanisms (31-33). One is a direct competition process in which the repressor protein inhibits binding of the 30S to the SD sequence at the RNA, either by steric hindrance, or by modifying RNA conformation in such a way that the SD sequence becomes inaccessible to the ribosome. In an alternative mechanism, called entrapment (34-36), the repressor protein allows the 30S subunit to bind to RNA, but traps it into an inactive complex that cannot proceed to further steps of the translation initiation process (Figure 7B). Whether Crc prevents or traps the initial binding of the 30S subunit on the mRNA remains unsolved. The footprinting assays indicate that Crc neither modifies the 2D structure of the leader part of *alkS* mRNA nor stabilizes the hairpin motif that may sequester the ribosome binding site. Hence, it seems likely that Crc poses a direct steric hindrance on ternary complex formation. The crystal structure of several ribosomal complexes shows that ~30 nt of the mRNA wrap around the neck of the 30S subunit (37-39). It was further proposed that, in the first step of the initiation, the mRNA binds to the 30S by establishing contacts between the SD sequence and the 3'-end of 16S rRNA on the 30S subunit platform. The following step involves a simultaneous positioning of the initiation codon into the P site to form the codon-anticodon interaction, and of the 3'-end

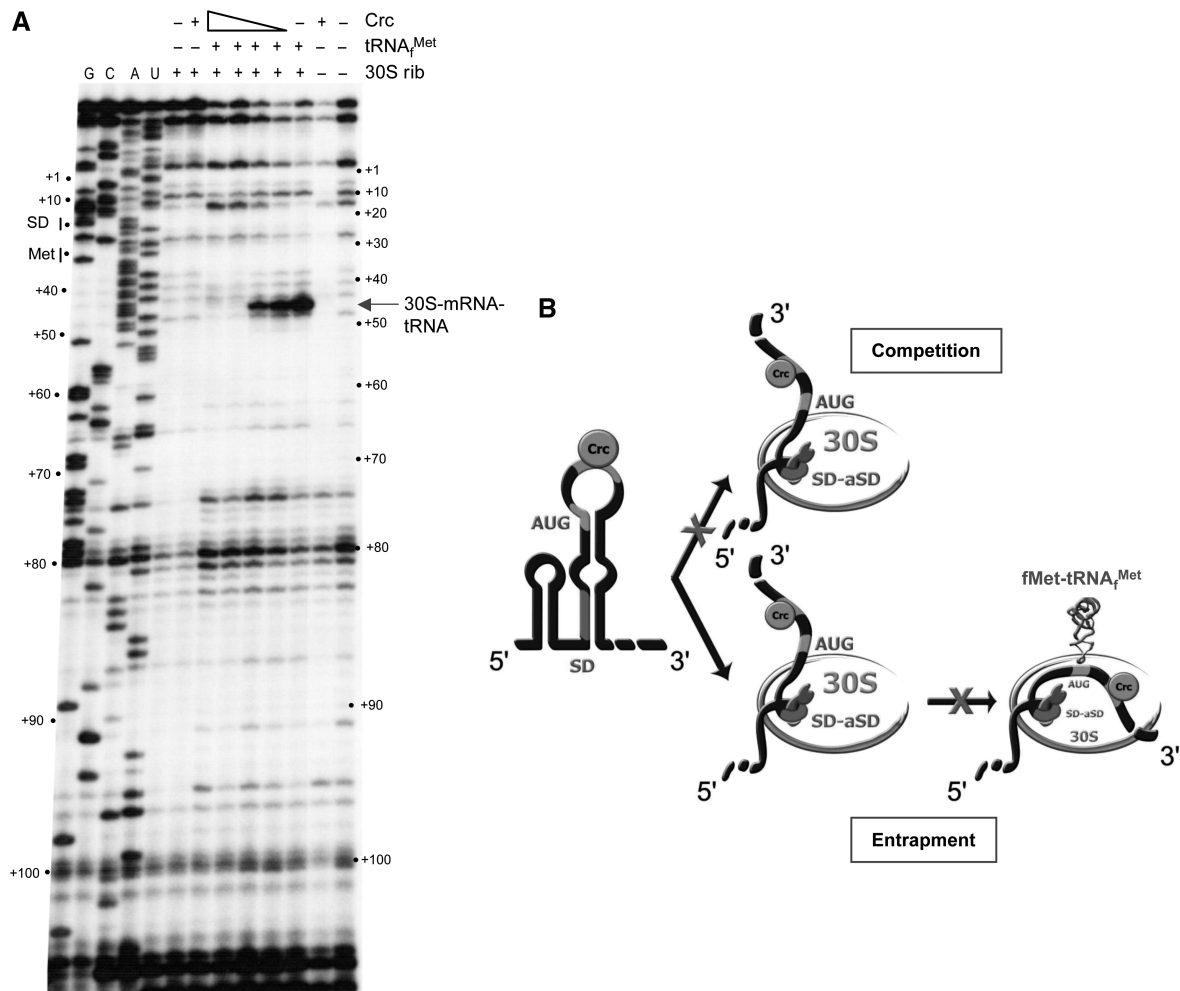


Figure 7. Crc binding to *alkS* mRNA prevents the formation of the ternary ribosomal initiation complex. (A) Toeprinting analysis showing the effect of Crc on the formation of the ternary complex formed between *alkS* mRNA, *E. coli* ribosomal 30S subunit and the initiator tRNA^{Met}. The unlabelled *alkS* mRNA (−37 to +200) was incubated in the absence (−) or in the presence of increasing concentrations of Crc (16 nM, 80 nM, 400 nM or 2 μM), tRNA^{Met}, or 30S ribosomes, as indicated. The primer extension products were resolved on a 12% urea–polyacrylamide gel, side by side with sequence ladders (lanes G, C, A, U). The toeprint corresponds to tRNA^{Met}-mRNA-30S complex that stops the elongation of reverse transcriptase (16 nts downstream the A of the AUG initiation codon). Positions relative to *PalkS2* transcription start sites are indicated on the sides of the gel. (B) Possible models of the effect of Crc on the formation of the ribosomal initiation complex. The formation of the 30S initiation complex involves as the first step the docking of mRNA structure (formation of the SD helix), followed by the accommodation step that correctly positions the rest of the mRNA to promote stable codon–anticodon interactions in the P-site. Crc binds to the AU rich sequence downstream the AUG codon of *alkS* mRNA and prevents formation of the ternary complex by blocking the accommodation process. Two mechanisms are possible: the 30S subunit cannot access the mRNA bound to Crc (competition mechanism), or Crc blocks the 30S subunit into an inactive complex (entrapment complex) and prevents the accommodation step to promote the codon-anticodon interaction at the P site. The model of the mRNA binding to the 30S subunit is taken from ref. (37).

of the mRNA (36–39). In this scenario, it is tempting to propose that Crc would prevent the accommodation step required to correctly position the AUG codon and the 3'-end of the mRNA into the normal ribosomal track. As a consequence, the codon–anticodon could not form (Figure 7B). A recent systematic antisense interference with 30S subunit binding to mRNA revealed that masking of the coding sequence down to the fifth codon, leaving the SD and the AUG codon unpaired, strongly inhibits the formation of the ternary initiation complex (40). In addition, the *Salmonella typhimurium* non-coding RNA (ncRNA) RybB represses the translation of *ompN* mRNA by forming a short duplex with the coding

sequence just downstream the AUG codon (40,41). Therefore, as for ncRNAs, regulatory proteins also prevent formation of the active initiation ribosomal complex by direct masking of the coding sequence close by to the AUG codon. We note, however, that Crc may not work in the same way with all target mRNAs, the mechanism differing depending on the location of the Crc-binding site relative to the AUG start site, and on mRNA structure. In *benR* mRNA, the Crc-binding site is located just upstream the SD sequence. Of interest, the S1 ribosomal protein in Gram-negative bacteria is known to bind to an unpaired AU rich motif located in the leader regions of mRNAs, i.e. upstream the SD.

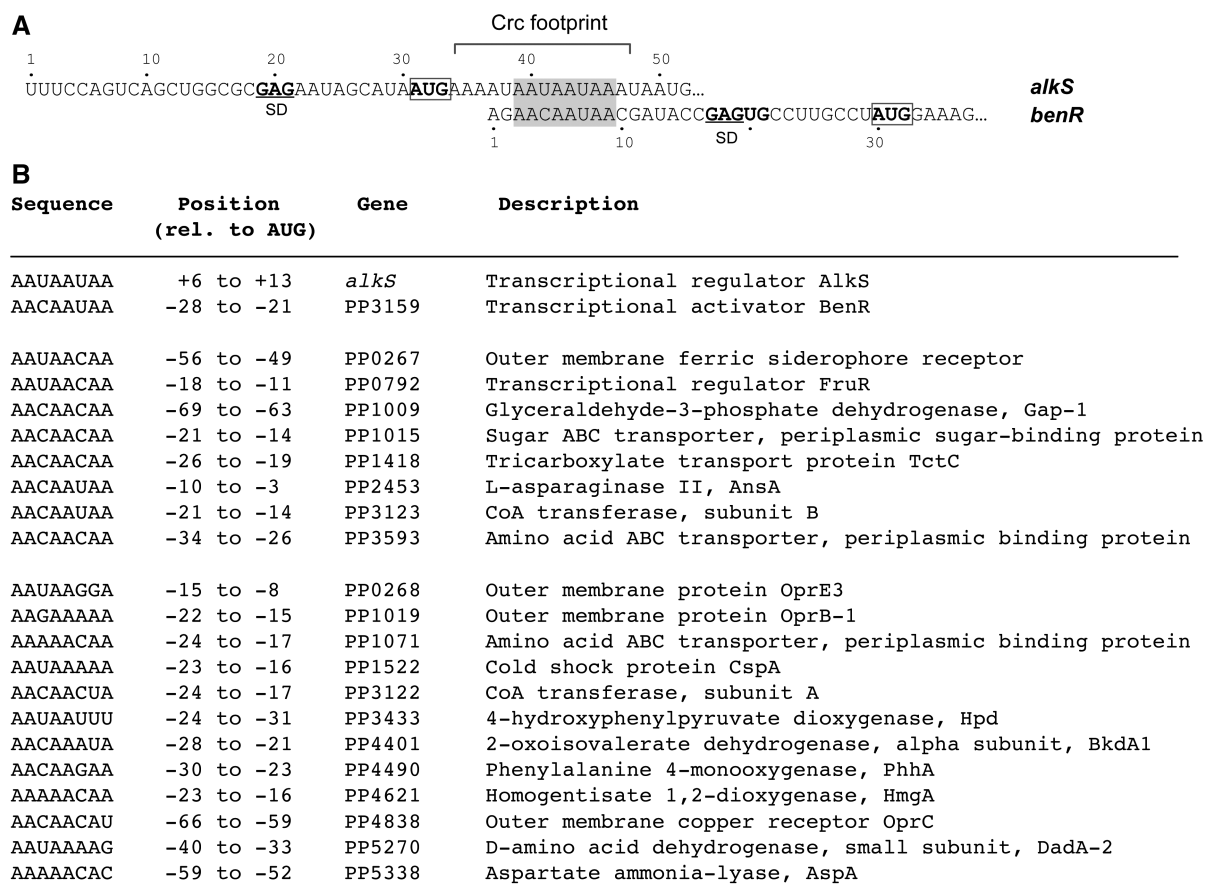


Figure 8. Identification of sequences similar to the *alkS* and *benR* Crc-binding sites in the proximity of the AUG start site of genes that are repressed by Crc. (A) Alignment of the 5' regions of *alkS* and *benR* mRNAs. The AUG initiation codon is boxed. The Crc binding site at the *alkS* mRNA is indicated. The nucleotides of the *alkS* and *benR* Crc binding sites that show sequence similarity are shaded. (B) Sequences similar to the Crc binding region common to *alkS* and *benR* mRNAs (shaded in panel A) found in the proximity of the AUG start site of known Crc-regulated genes; the position relative to the AUG start site, as well as the name and description of the genes, is indicated. The potential Crc-targets are divided into two classes, eight of them carry the consensus sequence AAYAAYAA and all others contain a divergent consensus rich in A residues. The transcription start sites are only available for *phhA* (44), *hpd* (45) and *bkdA1* (9) mRNAs, and the presumed Crc-target lies within the transcribed region.

This r-protein facilitates the initial ribosome binding of structured mRNAs, or of mRNAs having weak SD sequences as for *alkS* and *benR* mRNAs (42). Hence, in that particular case, Crc may prevent the initial ribosome binding to the *benR* mRNA by competing with ribosomal protein S1.

Several bacterial proteins can inhibit translation initiation by binding to the mRNA leader sequences (31–33). Translational regulation is believed to provide a fast response to changes in environmental or physiological conditions, and is therefore complementary to other regulatory mechanisms. The mechanisms of catabolite repression control described to date typically inhibit either the uptake of the non-preferred substrates by blocking the relevant permeases through processes based on protein–protein contacts (inducer exclusion), or the transcription initiation of the genes involved in the catabolism of the non-preferred compounds (2,3). The results presented in this work, together with previous evidence (14,15 and references therein), show that a key catabolite repression control network from Pseudomonads ultimately relies on

regulating translation initiation. This is in contrast to what has been found for other model bacteria like *E. coli* or *B. subtilis*, and may perhaps be related to the extreme metabolic versatility of Pseudomonads.

ACKNOWLEDGEMENTS

The authors are grateful to E. Martínez-Salas and M. Yusupov for helpful discussions and to L. Yuste and AC. Helfer for excellent technical assistance. We are indebted to K. Weeks for providing 1M7 reagent and advices for performing the chemical reactions and quantification of the data. R.M. was holder of a short-term fellowship from the European Molecular Biology Organization (ASTF 224.00-2008).

FUNDING

Spanish Ministry of Science and Innovation (BFU2006-00767/BMC and BFU2009-07009/BMC) and Instituto de Salud Carlos III (Spanish Network for the Research in

Infectious Diseases; REIPI RD06/0008) to F.R. Centre National de la Recherche Scientifique (CNRS) and the French National Agency for Research (ANR-07-blanc-0351-02) to P.R. Funding for open access charge: Grants from the Spanish Ministry of Science and Innovation (BFU2006-00767/BMC and BFU2009-07009/BMC to F.R.).

Conflict of interest statement. None declared.

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