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Involvement of a Putative Cyclic AMP Receptor Protein (CRP)-Like Binding Sequence and a CRP-Like Protein in Glucose-Mediated Catabolite Repression of *thn* Genes in *Rhodococcus* sp. Strain TFB

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Glucose catabolite repression of tetralin catabolic genes in *Rhodococcus* sp. strain TFB was shown to be exerted by a protein homologous to transcriptional regulators of the cyclic AMP receptor (CRP)-FNR family. The protein was detected bound to putative CRP-like boxes localized at the promoters of the *thnA1* and *thnS* genes.

Carbon catabolite repression (CCR) is a regulatory mechanism that ensures the utilization of the most favorable carbon and energy source present in the growth medium. CCR has been studied extensively in model bacteria such as *Escherichia coli* and *Bacillus subtilis* (3) and in *Pseudomonas putida* (8), a well-known degrader of aromatic compounds, revealing that CCR can be exerted by quite different molecular mechanisms. Several reports have described CCR of aromatic compound degradation in the genus *Rhodococcus* and proposed the involvement of CRP-like or glucose kinase-like proteins (1, 6). However, nothing is known about the molecular mechanism by which this regulatory phenomenon occurs in rhodococci.

Rhodococcus sp. strain TFB can use a variety of aromatic compounds as sole carbon and energy sources. CCR operates on the use of some of these molecules, such as tetralin (10), but not on others, such as phthalate (9). In this study, we analyzed the components involved in glucose-mediated CCR of the tetralin degradation genes of *Rhodococcus* sp. strain TFB.

Genes involved in tetralin catabolism in *Rhodococcus* sp. strain TFB are organized in one regulatory and two structural operons that are divergently transcribed (10) (Fig. 1A). *In silico* analysis of the *thnA1* promoter region (which is identical to the *thnA1′* promoter region [10]) revealed a palindromic DNA sequence (CTGTGT-N6-TCACAG) (Fig. 1B) similar to the *E. coli* CRP binding site (AAATGTGA-N6-TCACATTT) (12) that overlapped the −10 region and transcription start point (TSP). This sequence, designated as a putative CRP-binding box, is also found in the promoter regions of rhodococcal genes involved in the degradation of other aromatic compounds, for example, in the promoter region of the *catABC* operon of *Rhodococcus erythropolis* CCM2595 (AJ605581) (11), which is involved in catechol degradation. Transcription of *catABC* is controlled by CatR, an IclR-type repressor, and is subject to CCR by succinate by an unknown mechanism.

To study the role of this putative CRP-binding box in *thnA1* transcription, nucleotides of the palindromic sequence likely to be critical for CRP binding (determined by comparison with the crit-

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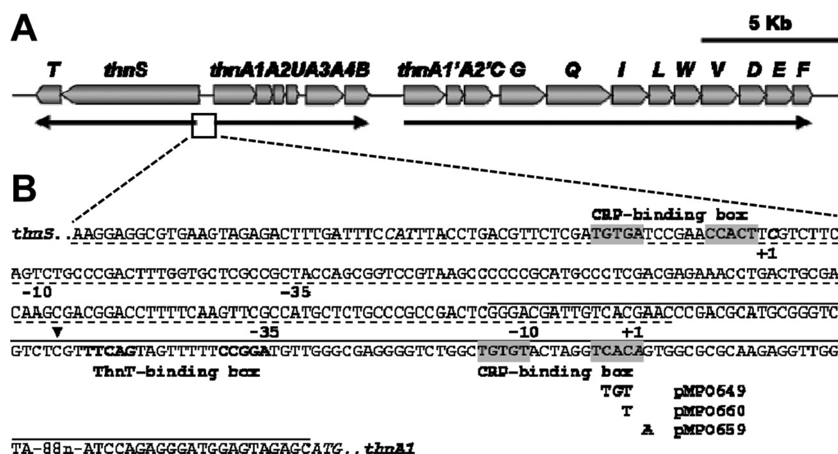

FIG 1 Tetralin degradation genes in *Rhodococcus* sp. strain TFB and promoter analysis. (A) Arrangement of *thn* genes in TFB. Transcription units are indicated by arrows. (B) Sequences of the TFB *thnA1* and *thnS* promoters and locations of the putative CRP-binding boxes. Nucleotide changes in the different promoter probe plasmids are indicated by boldface type. Biotinylated DNA fragments used in the binding assays for *thnA1* and *thnS* are shown by solid and dotted lines, respectively. An inverted triangle indicates the beginning of the *thnA1′* duplication.

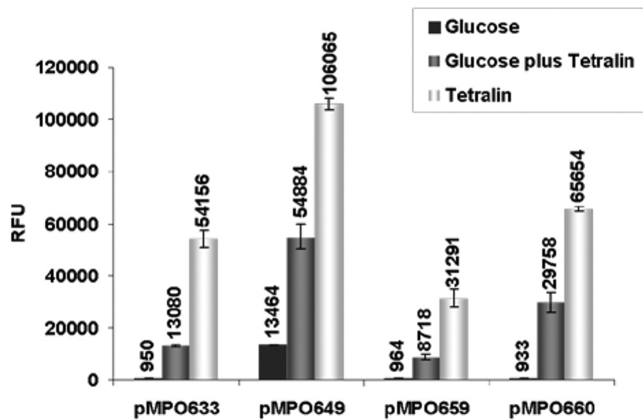

FIG 2 Fluorescence emitted from the *thnA1*::GFP translational fusions. pMPO633 is a *thnA1* wild-type promoter; pMPO660 carries a C-to-T substitution at the −1 position; pMPO649 carries a substitution of three nucleotides at positions −3, −2, and −1 (CAC to TGT); and pMPO659 bears an A-to-G substitution at position +2. RFU, relative fluorescence units. Three independent biological replicates were measured.

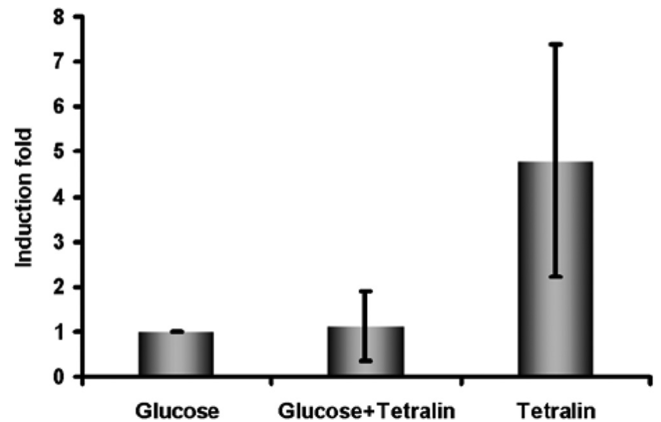

FIG 3 Transcription of *thnS* measured by RT-qPCR. *thnS* expression under different growth conditions is compared to levels of expression in glucose-grown cultures. The absolute quantity of *thnS* transcription in glucose alone is 0.451 ± 0.147 ng. Data are from three biological replicates for each condition.

ical nucleotides for CRP binding in *E. coli*) were mutated by PCR (Fig. 1B) and the resulting *thnA1* promoter sequences cloned into pMPO634, a replicative plasmid in TFB constructed to generate green fluorescent protein (GFP) translational fusions (10). pMPO633 contains *gfp* fused to the wild-type *thnA1* promoter, pMPO660 contains a C-to-T transition at the −1 position, pMPO649 contains a substitution of three nucleotides (CAC is replaced by TGT) at positions −3, −2, and −1, and pMPO659 bears an A-to-G transition at position +2 and was used as a control for mutations close to the putative CRP-binding site. These plasmids were introduced into TFB by electroporation, and GFP fluorescence was measured in cells grown in minimal medium containing tetralin (vapor phase), 20 mM glucose, or both carbon sources. Figure 2 shows that all of the mutated promoters were induced, though at different levels, in TFB cells grown with tetralin as the only carbon and energy source. Tetralin-plus-glucose-grown cells carrying pMPO633 (wild type) or pMPO659 (position +2 mutated) showed, as expected, about a 75% reduction in fluorescence compared with tetralin-grown cells carrying the same plasmids (Fig. 2), thus confirming CCR. However, GFP fluorescence from tetralin-plus-glucose-grown cells harboring pMPO660 or pMPO649 was only 50% of their respective maximal levels in the presence of tetralin alone. This indicated that CCR exerted by glucose was partially relieved by the mutations in these plasmid constructs and, therefore, that the mutated nucleotides play a role in glucose-mediated carbon catabolite repression. Furthermore, expression of the gene fusion in pMPO649 (containing the CAC-to-TGT substitution) in glucose-grown cells was 10-fold higher than that in the wild type and strains with other mutated *thnA1* promoter fusions, suggesting that these three substitutions also affected the basal level of *thnA1* expression.

Surprisingly, none of the mutations resulted in complete derepression of *thnA1* in the presence of glucose. It is possible that substitution of these conserved nucleotides did not fully impair CRP binding or that some degree of CCR is still possible in these mutants even in the absence of CRP binding. Alternatively, and perhaps more likely, this could reflect glucose-mediated CCR of the ThnST two-component transcriptional activator system required for activation of *thnA1* transcription (10). Quantitative reverse transcription-PCR (RT-qPCR) analysis (Fig. 3) indicated that the level of *thnS* transcription is very low (consistent with a level of fluorescence from a *thnS*::*gfp* fusion that is indistinguishable from background levels [data not shown]) and similar in glucose-grown and tetralin-plus-glucose-grown cultures but 4.8-fold higher in the presence of tetralin as the sole carbon source. Consistent with this result, a putative palindromic sequence similar to the *thnA1* CRP-binding box overlaps the TSP of *thnS* (Fig. 1B). An increase in *thnST* gene dosage resulted in higher *thnA1* expression (10), indicating that these transcriptional activators appear to be limiting in tetralin-grown cultures; thus, the 4.8-fold decrease in *thnS* expression in the presence of glucose might account for the partial CCR observed for the mutant promoters.

A similar situation was described for *Rhodococcus jostii* RHA1, in which both regulatory (*bphS1T1*) and biphenyl/ethylbenzene catabolic genes are subject to CCR by glucose (1). However, in this case CCR is not exerted directly on the *bphS1T1* promoter, which

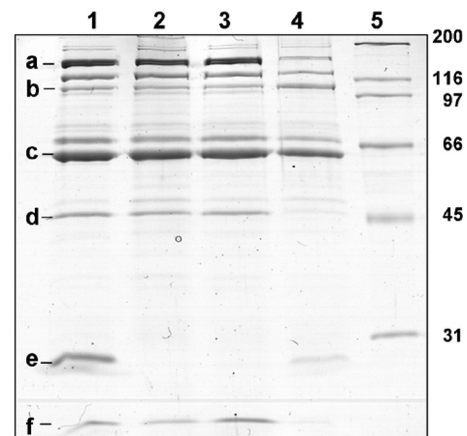

FIG 4 SDS-PAGE of proteins bound to the *thnA1* or *thnS* promoters. Lanes: 1, *thnA1* promoter; 2, mutated *thnA1* promoter from pMPO649 (CAC-to-TGT substitution at −3 to −1); 3, mutated *thnA1* promoter from pMPO660 (C-to-T transition at −1); 4, *thnS* promoter; 5, molecular size marker (sizes in kDa). Identified proteins are the β subunit of RNA polymerase (a), DNA polymerase I (b), biotin carboxylase (c), α subunit of RNA polymerase (d), CRP-FNR transcriptional regulator (e), and single-stranded DNA-binding protein (f). The gel was stained with EZBlue (Sigma), and proteins were manually excised and identified by MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry).

TABLE 1 Proteins bound to the *thnA1* and *thnS* promoter region identified by MALDI-MS

GenBank accession no.	Most similar protein	Organism	Score	Coverage (%)	M_w[a]	pI
gi|226363641	CRP-FNR type transcriptional regulator	*R. opacus* B4	335	92	26.95	8.12
gi|111025703	Biotin carboxylase	*R. jostii* RHA1	959	38	62.7	5.12
gi|111018959	β subunit RNA polymerase	*R. jostii* RHA1	306	38	128.5	4.91
gi|119366719	α subunit RNA polymerase	*R. jostii* RHA1	236	28	37.9	4.62
gi|226360133	DNA polymerase I	*R. opacus* B4	399	47	99.4	4.87
gi|111020420	Single-stranded DNA-binding protein	*R. jostii* RHA1	125	56	18	5.31

[a] Molecular weights are in thousands.

seems to be constitutive. Transcriptional read-through from the upstream *bphAa* promoter appears to be responsible for repression of *bphS1T1* transcription in the presence of glucose.

To identify putative transcriptional regulatory proteins acting directly on the *thn* promoter regions, DNA binding assays with TFB cell extracts were carried out. Biotinylated DNA fragments that extended from −92 to +132 of the wild-type and mutated versions of the *thnA1* promoter and from −150 to +68 of the *thnS* promoter (both with respect to TSP) were obtained by PCR amplification and bound to Dynabeads M-280 (Invitrogen) following the manufacturer's instructions. These were incubated with extracts from TFB cells grown on glucose (10). DNA binding assays (three independent experiments) were carried out at 4°C for 45 min, and bound proteins were identified by cutting bands from a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (*thnA1* and *thnS* promoters) (Fig. 4). Proteins with homology to rhodococcal biotin carboxylase, which is captured by streptavidin-coupled Dynabeads (2), RNA polymerase subunits, and DNA polymerase I were bound to the promoter fragments (Table 1). More interestingly, a protein homologous to a CRP-FNR-family transcriptional regulator from *Rhodococcus opacus* B4 (UniProt C1B9X5) was also identified, binding to both the *thnA1* and *thnS* promoter regions (Fig. 4). This protein is 100% identical (but with 19 amino acids missing at its N terminus) to the product of the ro04321 gene of *R. jostii* RHA1 (UniProt Q0S8M5), whose involvement in CCR in RHA1 has been proposed (1). This CRP-like protein shows the characteristic N-terminal effector domain and the C-terminal helix-turn-helix DNA binding domain of prokaryotic regulatory proteins belonging to the catabolite activation protein (CAP) family, which includes the CRP involved in CCR in many bacteria (4). Interestingly, binding of this protein to the mutated *thnA1* promoters was not observed (Fig. 4), consistent with a role for the putative CRP box in protein binding. In light of these results combined with the *thnS* expression data (Fig. 3), we propose that the TFB CRP-like protein acts as a transcriptional repressor of *thn* gene expression.

Concluding remarks. A CRP-like protein homologous to one proposed to be involved in CCR in *R. jostii* RHA1 (1) was identified in this work and found to be bound to the *thnA1* and *thnS* promoters of *Rhodococcus* sp. strain TFB grown in the presence of glucose. We also identified a putative CRP-binding sequence present in other rhodococcal promoters subjected to CCR. Although promoters mutated in the putative CRP-binding box still retained partial CCR, this could be explained by glucose-mediated CCR of the activation system ThnST. The positions of the CRP-binding boxes found at the *thnA1* and *thnS* promoters and the mutational analysis at the *thnA1* promoter indicate that the protein bound to this site could act as a transcriptional repressor of *thn* gene expression. A similar function has been described for GlxR, which represses genes involved in acetate metabolism (*aceA* and *aceB*) in glucose-grown *Corynebacterium glutamicum* (7) and binds to the consensus sequence TGTGANNTANNTCACA (5), which is very similar to the putative CRP-like binding sequence found in TFB.

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