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NOTES

Catabolite Repression Control by Crc in 2xYT Medium Is Mediated by Posttranscriptional Regulation of *bkdR* Expression in *Pseudomonas putida*

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The effect of growth in 2xYT medium on catabolite repression control in *Pseudomonas putida* has been investigated using the *bkd* operon, encoding branched-chain keto acid dehydrogenase. Crc (catabolite repression control protein) was shown to be responsible for repression of *bkd* operon transcription in 2xYT. BkdR levels were elevated in a *P. putida crc* mutant, but *bkdR* transcript levels were the same in both wild type and *crc* mutant. This suggests that the mechanism of catabolite repression control in rich media by Crc involves posttranscriptional regulation of the bkdR message.

The molecular mechanisms of catabolite repression have been well described in enteric bacteria, where enzymes of the phosphoenolpyruvate phosphotransferase system mediate catabolite repression control by regulation of cAMP concentration via adenylate cyclase activity (19). However, a similar mechanism does not appear to be present in *Pseudomonas* because adenylate cyclase activity and cAMP pools do not fluctuate with carbon source, nor does addition of cAMP relieve repression of catabolite responsive pathways (12, 18). The only protein thus far shown to be involved in catabolite repression in *Pseudomonas* is Crc of *P. aeruginosa*, but a function has not been identified (13). However, Crc does not appear to bind DNA (13), suggesting that it is not simply a DNA-binding negative regulator.

Crc is involved in catabolite repression of *P. putida* branched-chain keto acid dehydrogenase (BCKAD), glucose-5-phosphate dehydrogenase, and amidase by glucose and succinate in synthetic media (11). BCKAD is encoded by the four structural genes of the *bkd* operon, which is positively regulated by BkdR (15). BkdR is a homologue of Lrp (leucine-responsive protein), which is a global transcriptional regulator in *Escherichia coli* (4). However, pseudomonads and enteric bacteria live in complex media in nature and not in chemically defined media. Expression of *lrp* is downregulated in nutritionally rich media (6), which suggested that this might also be the case with *bkdR*. In this report, the effect of 2xYT medium on the expression of *bkdR* in wild type and in a *crc* mutant of *P. putida* was studied to determine if catabolite repression control of the *bkd* operon might be accomplished by controlling the level of BkdR in the cell.

Crc downregulates BCKAD activity in 2xYT. The wild-type strains of *P. putida* and *P. aeruginosa*, their *crc* mutants, and the complemented mutants (11) were grown to an A_{660} of ~0.6 in 100 ml of 2xYT plus 0.3% valine and 0.1% isoleucine (wt/vol) and then harvested; cell extracts were then prepared as described earlier (16). *P. putida* JS394 had five- to sixfold higher activity than either PpG2 or JS394 (pJRS196) (Table 1), and a similar result was obtained when BCKAD activity of PAO8020 was compared to the activities of PAO1 and PAO8020 (pPZ352). These results demonstrate that Crc is involved in catabolite repression control of BCKAD activity by 2xYT in both *P. putida* and *P. aeruginosa*. However, the BCKAD activities of the *crc* were much lower than that obtained in minimal media (11), indicating that something in addition to Crc is involved in catabolite repression control in synthetic medium.

It was interesting to investigate whether the *crc* mutants could be complemented with the heterologous *crc. P. aeruginosa* PAO8020 was transformed by triparental mating (9) with pJRS196, which contains *crc* from *P. putida* cloned in pUCPM19 (11). BCKAD activity in *P. aeruginosa* 8020 (pJR196) was similar to that seen in *P. aeruginosa* PAO1 and *P. aeruginosa* PAO8020(pPZ352) (Table 1). This demonstrates Crc has the same function in both species and that *P. aeruginosa* recognizes the *P. putida crc* promoter. Several attempts were made to complement the *P. putida crc* mutation with pPZ352, but for some reason, all these attempts were unsuccessful.

Crc reduces the level of BCKAD in 2xYT. BCKAD is a multienzyme complex with three components. The E1 component of *P. putida* BCKAD is an αβ heterotetramer (10), the structure of which has just recently been determined (1). The E2 component is a transacylase (2), and the E3 component is a specific lipoamide dehydrogenase (3). In mammalian cells, BCKAD activity is regulated by a posttranslational modification: E1α contains two phosphorylation sites, and the phosphorylation state regulates activity of the complex (17). Although *P. putida* E1α is not phosphorylated (10), it is possible that catabolite repression of *P. putida* BCKAD activity could be the result of some other kind of posttranslational modification of BCKAD or could be the result of reduction in transcription of the *bkd* operon. To distinguish between these two possibilities first, Western blots with anti-E1α serum (10) were employed. *P. putida* PpG2, JS394, and JS394(pJRS196), and *P.*

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TABLE 1. Effect of mutations in *crc* on repression of BCKAD activity by 2xYT in *P. putida* and *P. aeruginosa*

Strain[a]	Host genotype	Relevant plasmid genes[a]	BCKAD activity[b]
P. putida			
PpG2	*crc*$^+$		4
JS394	*crc* mutant		23
JS394(pJRS196)	*crc* mutant	*crc*$^+$ (*P. putida*)	2
P. aeruginosa			
PAO1	*crc*$^+$		3
8020	*crc* mutant		18
8020(pPZ352)	*crc* mutant	*crc*$^+$ (*P. aeruginosa*)	2
8020(pJRS196)	*crc* mutant	*crc*$^+$ (*P. putida*)	3

[a] Strains and plasmids were prepared under the growth conditions described earlier (11).

[b] BCKAD activity is given in nanomoles of NADH formed/min/mg of protein.

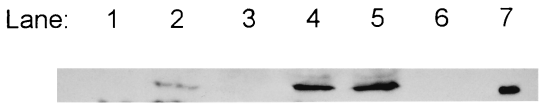

FIG. 2. Levels of BkdR in wild type and in the *crc* mutant of *P. putida*. These cultures were grown and harvested as in Fig. 1, and 200 μg of each cell extract was loaded onto an SDS–12% PAGE gel, blotted to Hybond-P membrane, and treated with anti-BkdR (14). More protein was used than in the experiment of Fig. 1 because of the low copy number of BkdR per cell. Hybond-polyvinylidene difluoride (PVDF) membranes (Amersham) were used in this experiment because PVDF has a better binding capacity for low-molecular-weight proteins. Proteins were separated by electrophoresis in an SDS–12% PAGE gel. Lane 1, *P. putida* grown in 2xYT plus 0.3% valine–0.1% isoleucine; lane 2, *P. putida* JS394 grown in 2xYT plus 0.3% valine–0.1% isoleucine; lane 3, *P. putida* JS394(pJRS196) grown in 2xYT plus 0.3% valine–0.1% isoleucine; lane 4, *P. putida* PpG2 grown in 0.3% valine–0.1% isoleucine synthetic medium; lane 5, *P. putida* PpG2 grown in 0.3% valine–0.1% isoleucine plus 40 mM succinate; lane 6, *P. putida* JS386 grown in 2xYT plus 0.3% valine–0.1% isoleucine; lane 7, 20 ng of purified BkdR.

aeruginosa strains PAO1, PAO8020, and PAO8020(pJRS196) were grown to an A_{660} of ~0.6 in 100 ml of 2xYT plus 0.3% valine and 0.1% isoleucine (wt/vol) and then harvested. Cell extracts were then prepared as described earlier (16). Five micrograms of protein was loaded onto a sodium dodecyl sulfate (SDS)–8.5% polyacrylamide gel electrophoresis (PAGE) gel, blotted to Hybond-enhanced chemiluminescence (ECL) membrane, and treated with anti-E1α serum. As seen in Fig. 1, E1α protein levels were greatly increased in the *crc* mutants *P. putida* JS394 and *P. aeruginosa* PAO8020 compared to the other four strains. The increase in E1α reflected the increased BCKAD activities found in these extracts (Table 1). Therefore, repression of BCKAD activity by Crc is due to a reduction in the amount of BCKAD. *P. putida* PpG2 grown in glucose minimal medium had no BCKAD activity, nor could E1α be detected by Western blots.

Crc regulates the level of BkdR produced in 2xYT. To characterize the role of BkdR in catabolite repression of BCKAD activity, Western blots with anti-BkdR serum were used to measure BkdR levels in wild type and the *crc* mutant of *P. putida* in grown in 2xYT plus valine-isoleucine and valine-isoleucine synthetic media. Then, 200 μg of cell extracts from *P. putida* PpG2, JS394, and JS394(pJRS196) grown in 2xYT plus 0.3% valine–0.1% isoleucine, along with *P. putida* PpG2 grown in 0.3% valine–0.1% isoleucine synthetic medium (16) alone or with 40 mM succinate, were loaded on an SDS–12% PAGE gel, blotted to Hybond-P membrane, and treated with anti-BkdR serum. As seen in Fig. 2, *P. putida* JS394 grown in 2xYT plus valine-isoleucine had higher levels of BkdR than either PpG2 or JS394(pJRS196) grown under the same conditions. This result demonstrates that Crc plays a major role controlling the level of BkdR in 2xYT and suggests that relief of catabolite repression control in *crc* mutants is due to a higher level of BkdR. BkdR levels of *P. putida* PpG2 were much higher in minimal media than in 2xYT (lanes 4 to 5 of Fig. 2), corresponding to the higher BCKAD activity in minimal medium (11). Also, the amount of BkdR was not repressed when succinate was added to the inducing medium, suggesting a different kind of control in minimal media. No BkdR was detected in *P. putida* JS386 which contains a *bkdR-lacZ* translational fusion (14).

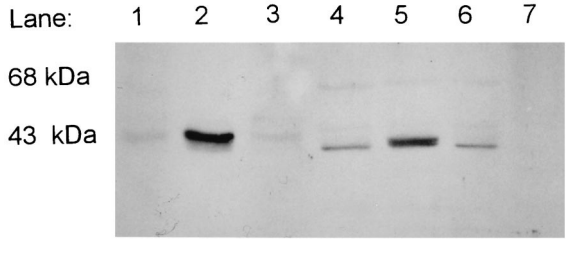

FIG. 1. Western blot with anti-E1α serum (10) of *crc* mutants grown in 2xYT plus valine-isoleucine. The extracts in lanes 1 to 6 were from cultures grown in 2xYT plus 0.3% valine and 0.1% isoleucine (wt/vol); the extract in lane 7 was from a culture grown in glucose minimal medium. Each lane contained 5 μg of protein. Lane 1, *P. putida* PpG2; lane 2, *P. putida* JS394; lane 3, *P. putida* JS394(pJRS196); lane 4, *P. aeruginosa* PAO1; lane 5, *P. aeruginosa* PAO8020; lane 6, *P. aeruginosa* PA8020(pJRS196); lane 7, *P. putida* PpG2 grown in glucose. All cultures were grown to an A_{660} of between 0.6 and 0.8, and cell extracts were prepared as described before (16). Electrophoresis was done in an SDS–8.5% PAGE gel. Western blots were screened by using the ECL-Western blotting analysis system (Amersham Pharmacia Biotech) with Hybond-ECL nitrocellulose membranes according to the manufacturer's instructions. To determine whether the ECL detection method was quantitative, increasing amounts of PpG2 grown in valine-isoleucine-lactate medium were loaded on a gel and used for Western blotting with anti-E1α serum. The blot was scanned with a Molecular Dynamics densitometer, and pixel values versus micrograms of protein were graphed and shown to be a linear plot (data not shown).

Repression of BCKAD activity by 2xYT involves posttranscriptional regulation of *bkdR* expression. Since both BCKAD and BkdR levels are elevated in the JS394 grown in 2xYT supplemented with valine and isoleucine (Fig. 1 and 2), mRNA levels of *bkdR* and *bkdA1*, which encodes E1α, were of interest in determining the mechanism of action of Crc in repression by 2xYT. *P. putida* PpG2, JS394, and JS394(pJRS196) were grown in 2xYT plus valine-isoleucine medium. *P. putida* JS382 (15), a mutant with a deletion in *bkdR*, which does not produce *bkd* operon mRNA, was grown under the same conditions for use as a negative control. At mid-log phase, total RNA was purified from each culture. Five micrograms total RNA was transferred to a GeneScreen Plus membrane by means of a vacuum suction HYBRI-SLOT Filtration Manifold (Life Technologies). The membrane was prehybridized for 1 h at 42°C and then hybridized overnight with 0.5 μCi of *bkdA1* or *bkdR* mRNA probes at the same temperature. After a washing at 65°C, the membrane was exposed to a Phosphor Screen (Molecular Dynamics) overnight. The membrane was stripped by boiling for 30 min in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 1% SDS and then prehybridized and hybridized as described above with 0.3 μCi of 5′-end-labeled 16S RNA probe. The adjusted pixel value for each mRNA was obtained by normalizing each slot pixel value to its 16S RNA value and then averaging the duplicates and subtracting the average of the normalized *P. putida* JS382 pixel

TABLE 2. Analysis of mRNA levels and BCKAD activity in wild type and *crc* mutant of *P. putida*

P. putida strain[a]	*bdkA1* message[b]	*bkdR* message[b]	BCKAD activity[c]
PpG2	38	10	4
JS394	165	5	23
JS394(pJRS196)	38	7	2

[a] See Table 2 for genotypes.
[b] mRNA levels are expressed in pixels.
[c] BCKAD activity is given in nanomoles of NADH formed/min/mg of protein.

values. After hybridization and a washing, the membrane was exposed to a Phosphor Screen for 1 to 2 h.

Probes for *bkdA1* and *bkdR* were generated from PCR fragments and were labeled with Ambion's Prime-A-Probe kit; a larger PCR fragment was primed internally with a third primer, so that the single-stranded DNA (ssDNA) probe could be gel purified away from the PCR product. A 286-bp *bkdA1* PCR fragment was amplified with primers S73 (nucleotides [nt] 1615 to 1641) and S114 (nt 1901 to 1883). This fragment was primed internally with S87 (nt 1698 to 1681), yielding an 83-base ssDNA probe complementary to the *bkd* operon mRNA. A 511-bp *bkdR* PCR fragment was amplified with primers S88 (nt 916 to 935) and S58 (nt 1427 to 1411). This fragment was primed internally with two different primers: S40 (nt 1287 to 1300) produced a 140-base ssDNA probe, while S39 (nt 1022 to 1036) produced a 405-base probe, both complementary to *bkdR* mRNA. For the 16S RNA probe, the primer S179 (nt 33 to 10), which is complementary to the mRNA, was 5′-end-labeled with T4 kinase. The accession numbers for each sequence are as follows: *bkd* operon, M57613; and 16S RNA, D85995.

Two identical blots were prepared by loading each RNA sample in duplicate onto a slot blot. These blots were first probed with radiolabeled ssDNA probes to either *bkdR* or *bkdA1* mRNA. After this, the blots were stripped and reprobed with a radiolabeled 16S RNA oligonucleotide to normalize blots for the amount of RNA loaded.

The levels of *bkdA1* mRNA were typically four- to sixfold higher in *P. putida* JS394 than in PpG2 or JS394(pJRS196) (Table 2). This result, taken together with the BCKAD assays and the Western blots of E1α levels, indicates that regulation of *bkd* operon expression by 2xYT occurs by reducing transcription. However, *bkdR* mRNA levels in JS394 were always similar to or slightly lower than the levels seen in PpG2 and JS394(pJRS196) (Table 2). This suggests that the mechanism of regulation of *bkdR* expression by 2xYT occurs at a posttranscriptional level.

The data in our related study demonstrated that Crc was involved in catabolite repression control of BCKAD, glucose-6-phosphate dehydrogenase, and amidase in synthetic media (11). In the present study, it has been shown that the amount of BkdR was elevated in the *crc* mutant, JS394 (Fig. 2) but that the amount of *bkdR* mRNA was unchanged (Table 2), which suggests that Crc acts posttranscriptionally in controlling BkdR levels. In contrast, *bkdA1* mRNA, BCKAD activities (Table 2) and E1α protein levels (Fig. 1) were all elevated in the *P. putida crc* mutant, indicating that expression of the *bkd* operon was regulated at the transcriptional level. It was also shown in (11) that *lacZ* expression was increased two- to threefold in the mutant with transposon-inactivated *crc* (*P. putida* JS391) carrying a *bkdR-lacZ* translational fusion. However, *lacZ* is inserted after the 44th amino acid codon of BkdR (14), and this transcript would look very different to Crc than the normal *bkdR* message.

Crc shares sequence similarity with a group of DNA repair enzymes, although no endo- or exonuclease activity has been identified (13). It is possible that Crc's nuclease activity is very specific, such as acting only on secondary RNA structure, resulting in functional degradation of mRNA. Crc could effect posttranscriptional regulation of *bkdR* expression by affecting the efficiency of translation or the stability of functional mRNA. Two types of secondary structures are responsible for controlling mRNA stability: 5′ hairpins and 3′ hairpins. The 5′ untranslated region of the *E. coli ompA* transcript functions in vivo as a growth-rate-regulated mRNA stabilizer (7). The hairpin in this untranslated region is not only specific for the *ompA* gene but also confers stability when fused to other genes (5). The half-life of the mRNA was drastically reduced when the stem-loop structure was moved more than ten nucleotides away from the 5′ end (5). These results indicate that the stabilization provided by the hairpin is due to inhibition of endonuclease cleavage. Another secondary structure that can confer stability to a transcript is a 3′ hairpin. Stem-loop structures at the 3′ end of a transcript were originally thought to function only as ρ-independent transcriptional terminators, but more recently these structures have been shown to protect mRNA from degradation by the exonucleases RNase II and PNPase (8).

One possible explanation for the function of Crc in rich media is that there is a ligand in 2xYT which causes a conformational change in Crc, thereby activating it. Activated Crc would now have endonuclease activity which causes functional degradation of *bkdR* message. There is some support for this hypothesis, since Yuste et al. (20) showed that use of fresh Luria-Bertani medium resulted in catabolite repression of the *alk* operon of *Pseudomonas oleovorans*, whereas spent medium did not.

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