Role of the *ptsN* Gene Product in Catabolite Repression of the *Pseudomonas putida* TOL Toluene Degradation Pathway in Chemostat Cultures[▽]

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The *Pseudomonas putida* KT2440 TOL upper pathway is repressed under nonlimiting conditions in cells growing in chemostat with succinate as a carbon source. We show that the *ptsN* gene product IIA^{Ntr} participates in this repression. Crc, involved in yeast extract-dependent repression in batch cultures, did not influence expression when cells were growing in a chemostat with succinate at maximum rate.

Pseudomonas putida KT2440 *mt-2* is able to degrade and grow on aromatic substrates such as toluene and xylenes. The upper and *meta*-cleavage pathways required for complete degradation are coded by the TOL plasmid pWW0, where XylR is the master regulator of the concerted expression of the two pathways (21). The *xylR* gene is transcribed from two σ^{70} -dependent tandem promoters, P_{R1} and P_{R2} (Fig. 1). In the presence of substrates of the pathway, XylR protein becomes active (1) and induces transcription from the two σ^{54} -dependent promoters of this system, the upper-pathway promoter Pu and the *xylS* promoter P_{S1}. XylR binding sites to activate P_{S1} overlap the two *xylR* tandem promoters, thus repressing their expression and consequently its own synthesis (3, 21, 23).

XylR induction of the TOL pathway is silenced under several growth conditions. Repression is exerted at the transcriptional level, the catabolite repression targets being the σ^{54} -dependent promoters Pu and P_{S1} (8, 17). Initially, this was referred to as “exponential silencing” (4), because TOL induction was repressed during exponential growth in rich medium and derepressed in the late exponential phase (6, 16). In contrast to the previous observation, in cells growing on minimal medium in the exponential phase, TOL upper-pathway activation occurred immediately after the addition of a XylR effector. This difference was attributed to the presence of inhibitory compounds in the rich medium (17). In addition, some carbon sources (glucose, gluconate, α -ketogluconate, lactate and acetate) but not others (succinate, citrate, pyruvate, glycerol, fructose, and arabinose) inhibited activation of Pu in batch cultures (4, 14, 25). Finally, full repression of the TOL pathway was observed in cells growing in continuous culture at a nonlimiting rate ($\mu_{\max}$) at the expense of succinate (8). At a low growth rate, when an anabolic substrate (P, S, or N) was the growth-limiting factor (equivalent to a high-energy state in the cell) (12), the TOL degradation pathway was also repressed,

whereas when a catabolic substrate (C or O₂) was the growth-limiting factor (low energy state) (12), the pathway was functional (9). This suggests that catabolite repression is mediated by excess of carbon and/or energy rather than growth rate.

Some proteins have been involved in this process: IIA^{Ntr}, the *ptsN* gene product, contributes to Pu repression by glucose or gluconate in batch cultures through a mechanism that would involve the phosphorylation of the conserved residue His⁶⁸ (5). Conversely, IIA^{Ntr} did not influence Pu down-regulation during exponential growth on LB (5). These results pointed to differences in the genetic factors used by the cell to adapt to environmental conditions. However, we have recently shown that IIA^{Ntr} is also involved in the rich medium-dependent repression of Pu and P_{S1} (2). In addition, Crc, a protein involved in several catabolite repression mechanisms in *Pseudomonas* spp. (13, 20, 25, 26), also contributes to TOL σ^{54} -dependent promoter repression in rich medium. The role of Crc could be to “sense” the energy state and thus help the cell to launch an appropriate response (2).

Nevertheless, our knowledge of the genetic factors involved in TOL catabolite repression in continuous culture is limited. It is known that in batch cultures high concentrations of the preferred carbon source are required to produce catabolite repression (22). In contrast, repression in continuous cultures was observed with a lesser excess of the repressive carbon source, suggesting that the cell physiological state (i.e., nutritional excess) rather than the carbon source per se was the signal triggering the response. To clarify whether cells respond differently in terms of catabolite repression under optimal growth conditions, we investigated the extent to which TOL inhibition in cells growing in chemostat at a maximum growth rate (excess of all nutrients, including carbon; thus, a high-energy state) was affected by the inactivation of genes known to play a role in repression in batch culture.

We set up a number of continuous cultures of *P. putida* KT2440 (pWW0) and its *ptsN* and *crc* mutants (2). Custom-made reactors with working volumes of 100 ml of mineral salt medium (10) supplemented with 10 mM succinate, a primary carbon and energy source for pseudomonads (11), were used. Cultures were maintained at 30°C with a constant airflow. Cells were grown at a low dilution rate ($D = 0.05 \text{ h}^{-1}$, with succinate

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FIG. 1. pWW0 TOL plasmid upper-pathway regulation. The diagram shows stimulation by the XylR protein of the two TOL pathway σ^{54} -dependent promoters, Pu and P_{S1} . The TOL upper-pathway and regulatory genes *xylS* and *xylR* are shown as gray arrows. R is any substituent group in the aromatic ring. Elliptical and square boxes indicate the inactive and active forms of XylR, respectively. Plus and minus signs indicate transcription activation and repression by XylR, respectively. Black small boxes represent XylR binding sites to activate Pu and P_{S1} (UASs). Promoters are outlined as flags.

as the growth-limiting factor) to determine full induction level and at a high dilution rate ($D = 0.85 \text{ h}^{-1}$, with carbon excess) to set up catabolite repression conditions (8). Residual succinate concentrations at the high dilution rate were 1 to 6 mM, in similarity to previously reported values (10). At $D = 0.05 \text{ h}^{-1}$, no succinate was detected in the effluent. After 5 min induction with the nonmetabolizable effector *o*-xylene through the gas phase, 10-ml samples were taken for RNA isolation, primer extension assays, and routine analyses as described previously (2, 18).

Figure 2 compares the results of induction of Pu and P_{S1} at the two dilution rates. As shown before, transcription from the Pu and P_{S1} promoters in the wild-type strain was repressed under conditions of carbon excess ($D = 0.85 \text{ h}^{-1}$) compared to the results seen with cells growing with succinate as the growth-limiting factor ($D = 0.05 \text{ h}^{-1}$) (8). Pu transcription was reduced to about 10% of the level in a carbon and/or energy-limited medium, while P_{S1} transcription was reduced to about 25% of the values obtained under conditions of succinate limitation. Differences between the Pu and P_{S1} promoter regions (21) may account for the different repression levels seen in the two promoters under the same growth conditions.

Analysis of the σ^{70} -dependent *xylR* tandem promoter transcription is a useful tool to indirectly track the efficiency of P_{S1} transcription. As mentioned above, XylR activation of P_{S1} blocks P_{R1} and P_{R2} transcription through binding to its upstream activation sites (UASs) overlapping P_{R1} and P_{R2} promoters (21). Therefore, when P_{S1} transcription is favored, P_{R1} and P_{R2} transcription is significantly reduced (3, 15). Figure 3 shows that in the wild-type strain, *xylR* promoter transcription was practically doubled in cells growing under conditions of carbon excess compared to the results seen with cells growing under conditions of carbon and/or energy limitation. This confirms a lower occupancy of XylR UAS in P_{S1} at high growth rates, in good correlation with the observed decrease in P_{S1} activity.

Repression of Pu transcription in the *ptsN* mutant when the culture was subjected to carbon and/or energy excess was lower

FIG. 2. Repression levels of Pu and P_{S1} promoters from *P. putida* (pWW0) and its *ptsN* and *crc* mutants. Cells were grown in continuous culture, at $D = 0.05 \text{ h}^{-1}$ (carbon limitation) and $D = 0.85 \text{ h}^{-1}$ (carbon excess), when *o*-xylene was added in the gas phase during 5 min. The presence of messengers was analyzed using reverse primer extension of equal amounts (10 or 20 μg) of total RNA and the corresponding labeled specific oligonucleotide (2). Samples were run in urea sequencing gels, which were exposed to a phosphor screen (Fuji Photo Film Co. Ltd.) for 5 to 12 h. Phosphor screens were scanned with a phosphorimaging instrument (Molecular Imager FX; Bio-Rad), and data were quantified with Quantity One software (Bio-Rad). The graph shows the mRNA levels of Pu (A) and P_{S1} (B) promoters in the three strains at $D = 0.85 \text{ h}^{-1}$ (repression conditions) as a percentage of the levels observed in each strain at a low growth rate ($D = 0.05 \text{ h}^{-1}$, full induction levels). (C) Representative urea-polyacrylamide gel electrophoresis results corresponding to mRNA-derived cDNA from the two promoters at each growth rate are shown. wt, wild type.

than in the wild-type strain. In this mutant, Pu transcription was reduced by about 50% compared to transcription in the nonlimited medium. A similar behavior was observed for P_{S1} transcription, although P_{S1} derepression was less apparent because of the weaker repressive effect on this promoter in the wild type (Fig. 2). As expected, the increase in P_{R1} and P_{R2} transcription was smaller when P_{S1} transcription was more markedly enhanced under nonlimiting growth conditions (Fig. 3).

When we analyzed transcription of Pu and P_{S1} in the *crc* mutant as described above, we found no significant difference from the wild-type strain results (Fig. 2). Accordingly, P_{R1} and P_{R2} transcription remained unchanged (Fig. 3). In the light of these results, a question mark hangs over the role of Crc in TOL catabolite repression in batch cultures when glucose is the repressor agent. Crc has been shown to contribute to TOL regulation in rich medium-mediated repression (2), but no data are available on its potential role in glucose-mediated repression in batch cultures. To investigate this issue, we compared the β -galactosidase activity of wild-type and *crc* mutant strains carrying a Pu::lacZ fusion and *xylR* gene in the pS10 plasmid (2) in the presence of 75 mM (1.5% [wt/vol]) glucose in the medium. The *P. putida ptsN* mutant was used as a

FIG. 3. Level of mRNA derived from the *P_{R1}* and *P_{R2}* promoters from *P. putida* (pWW0) and its *ptsN* and *crc* mutants grown in continuous culture in response to *o*-xylene at *D* = 0.05 h⁻¹ (carbon limitation) and *D* = 0.85 h⁻¹ (carbon excess). The presence of messengers was analyzed by reverse primer extension of equal amounts (10 or 20 μ g) of total RNA with the corresponding labeled specific oligonucleotide (2). Samples were run in urea sequencing gels, which were exposed to a phosphor screen (Fuji Photo Film Co. Ltd.) for 5 to 12 h. Phosphor screens were scanned with a phosphorimaging instrument (Molecular Imager FX; Bio-Rad). Data were quantified with Quantity One software (Bio-Rad). (A) The diagram at the top of the figure represents the *xylR/xylS* intergenic region (15). (B) The graph shows the mRNA levels of *P_{R1}* and *P_{R2}* promoters in the three strains at *D* = 0.85 h⁻¹ as a percentage of the levels observed in each strain at a low growth rate (*D* = 0.05 h⁻¹). (C) Representative urea-polyacrylamide gel electrophoresis results corresponding to mRNA-derived cDNA from the two promoters at each growth rate are shown. *wt*, wild type.

positive control of derepression of the Pu promoter (5). Fresh mineral salt medium cultures (5) were divided in two fractions. One was supplemented with both the XylR inducer *o*-xylene and with 1.5% glucose; only the inducer was added to the second fraction. When cultures reached the exponential phase, β -galactosidase activity was determined (19) (Fig. 4). Pu promoter expression was repressed in the presence of 1.5% (wt/vol) glucose in the wild-type strain *P. putida* KT2440 (4), but, as expected, this repression was weaker in the *ptsN* mutant (5). In the *crc* mutant, glucose repression of Pu was similar to wild-type results. This finding shows that despite its role in Pu repression in rich medium (2), Crc does not participate in the Pu repression caused by an excess of carbon either in continuous or in batch cultures (Fig. 4). It has been shown that Crc levels vary with growth medium composition. While Crc expression significantly increases when yeast extract is added to cells growing on glucose minimal medium (2), its levels are low

FIG. 4. Effect of glucose on Pu expression. β -galactosidase activity (in Miller units [MU]) was measured in *o*-xylene-induced batch cultures of *P. putida* strain KT2440 and its *ptsN* and *crc* mutants carrying the pS10 plasmid with Pu fused to the *lacZ* gene and the regulator gene *xylR* (2) in the presence or absence of glucose.

in continuous cultures with succinate excess (24). This suggests that the role of Crc in catabolite repression of the TOL plasmid is related to its concentration in the cell, which seems to be modulated by the composition of the medium (i.e., the carbon source). This is consistent with previous findings showing no contribution of Crc to succinate-dependent catabolite repression of the *P. putida* GPO1 alkane degradation pathway (7).

Taken together, these results help clarify some aspects of the processes controlling the toluene degradation pathway of the TOL plasmid pWW0 in *P. putida* KT2440. Bacterial growth in continuous culture helps maintain a physiological steady state at a selected growth rate in a closed culture system. In this study we analyzed how knockout of *ptsN* and *crc*, the two genes known to play an important role in TOL repression under batch culture conditions (2, 5), affected the intensity of repression of the TOL upper pathway in continuous culture with carbon and/or energy excess. Primer extension analyses showed that, in contrast with findings in batch cultures, the *ptsN* gene product but not Crc protein was involved in the phenomenon of TOL catabolite repression under these conditions. Subsequent analysis of Pu expression in the *crc* mutant grown in batch cultures confirmed that Crc did not play a role in TOL repression by carbon excess. These results point to a difference in the genetic factors used by the cell to adapt to different environmental conditions that cause TOL catabolite repression. We conclude that no single response mechanism is suitable for all environmental conditions; instead, cells may resort to more than one strategy to react appropriately to a changing environment. Catabolite repression triggered by carbon and/or energy excess and repression produced by some components of rich medium are sensed differently. In the first case, IIA^{Ntr} (PtsN), hence, a mechanism involving σ^{54} -dependent transcriptional machinery, plays a central role, while Crc protein seems to be involved only in the negative modulation of catabolic pathways when cells are growing exponentially on a complex rich medium.

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