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Induction and repression of the *sty* operon in *Pseudomonas putida* CA-3 during growth on phenylacetic acid under organic and inorganic nutrient-limiting continuous culture conditions

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Abstract

The effects of various nutrient-limiting conditions on expression of the *sty* operon in *Pseudomonas putida* CA-3 were investigated. It was observed that limiting concentrations of the carbon source phenylacetic acid, resulted in high levels of phenylacetyl coenzyme A (CoA) ligase activity, this was accompanied also by upper pathway styrene monooxygenase enzyme activity. The introduction of inorganic nutrient limitations, (nitrate, sulfate and phosphate), caused a dramatic reduction in detectable levels of phenylacetyl CoA ligase activity, particularly in the presence of the primary carbon source, succinate. Under these conditions it was no longer possible to detect styrene monooxygenase activity. Reverse transcription PCR analysis of total RNA, isolated under each of the continuous culture conditions examined, revealed that variations in the levels of enzyme activity coincided with altered patterns of corresponding *paaK* (phenylacetyl CoA ligase) and *styA* (styrene monooxygenase) gene expression. Transcription of the upper pathway regulatory sensor kinase gene *styS* was also observed to be growth condition-dependent. These observations suggest that induction/repression of the *sty* operon in *P. putida* CA-3, during growth on phenylacetic acid under continuous culture conditions, involves regulatory mechanisms coordinately affecting both the upper and lower pathways and acting at the level of gene transcription.

Keywords: Phenylacetic acid degradation; Phenylacetyl coenzyme A ligase; Styrene monooxygenase; Transcriptional regulation; Catabolite repression

1. Introduction

Batch culture (non-limiting growth) conditions provide a convenient method of assessing a microorganisms ability to degrade an aromatic compound. However, the data generated is typically inadequate to predict the organisms catabolic potential in nature. In its natural environment an organism is subjected to organic and inorganic nutrient limitation, which can dramatically affect the expression of its catabolic pathways [1]. Overcoming this dilemma is difficult due to the dynamic nature of the environment and the geographically dependent diversity in soil compositions, making exact duplication of natural environments an unrealistic objective. Continuous culturing of organisms offers a partial solution to this problem where, through careful manipulation of chemically defined media and subsequent dilution rates, one can emulate individual nutrient limitations, which may be encountered by a microorganism in nature.

This approach has been adopted in the past to study induction and repression of the toluene upper and lower degradative pathways, encoded by the TOL plasmid pWWO, under various nutrient-limiting conditions [2–4]. Previous work on the continuous culturing of *Pseudomonas putida* CA-3 by O'Connor et al. [5] assessed the effects of carbon, nitrogen and sulfur limitation, (C-, N- and S-lim, respectively), on the styrene-degrading ability of the strain in the presence and absence of primary carbon sources. This work was performed by monitoring two

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upper pathway enzyme activities, namely styrene oxide isomerase and phenylacetaldehyde dehydrogenase, and by measuring oxygen-uptake rates by washed whole cells, grown under various limiting conditions, when fed intermediates of styrene degradation.

It was the aim of this study, therefore, to further characterise regulation of the *sty* operon in *P. putida* CA-3 cells, during growth on the lower pathway substrate phenylacetic acid (PAA) in the presence of various growth-limiting conditions. Pathway expression levels were assessed via colourimetric whole cell-based assays specific for the first enzyme of both the upper and lower pathways, styrene monooxygenase (SMO) and phenylacetate-CoA ligase, (PAAK), respectively. Enzyme activities were found to be growth condition-dependent. To assess whether nutrient limitation affected gene expression or post-translational functioning of the pathway enzymes, reverse transcription (RT)-PCR analysis of the structural genes *paaK* (phenylacetylCoA ligase) and *styA* (SMO) was performed. Transcription of the histidine kinase sensor, *styS*, of the two-component, positive regulatory apparatus controlling upper pathway induction, was also monitored and found, as with all of the genes examined, to display growth condition-dependent patterns of expression. Finally, it was decided to investigate, for the first time, the effects of phosphorous limitation on regulation of the *sty* operon in *P. putida* CA-3.

2. Materials and methods

2.1. Media and buffers

Evans mineral salts media was used to support bacterial growth (25 mM NH_4Cl, 0.5 mM Na_2SO_4, 1 mM nitrilotriacetic acid, 0.3 mM $MgCl_2$, 5 μM $CaCl_2$, trace ammonium molybdate and an anti-sporulation agent, spore-opl (5 ml 0.25X/l media)). Following addition of the respective carbon source, the pH of the media was adjusted to 6.8, buffering was provided by 50 mM phosphate buffer. C-lim was achieved via addition of 10 mM succinate and/or 5 mM PAA. Non-limiting carbon conditions required 20 mM succinate and/or 10 mM PAA. Inorganic nutrient limitations were achieved by varying the concentrations of the respective salts. Harvested cells were washed at 0–4°C in 50 mM phosphate buffer.

2.2. Chemostat culture

The fermenters used were custom made with the culture vessel being shaped like an Erlenmeyer flask, with a total volume of 125 ml and a working volume of 100 ml as described previously [2]. Growth was maintained at a dilution rate of 0.05 l h^{-1} with air being supplied at 8 l h^{-1}. Stirring was performed magnetically at 500 rpm with a Teflon-coated stirring bar to ensure thorough aeration of the culture and rapid equilibration of fresh media. Cell density was measured via OD_{540} on a Beckman DU640 spectrophotometer. In order to assess substrate consumption, 5-ml samples from the chemostat were filtered through a 0.22-μm pore-size filter, to remove cells, and subjected to reversed-phase chromatography (Nucleosil 100 HD C18 125×3 mm) using a Hewlett-Packard HP1050 Ti HPLC coupled to a diode array detector (Hewlett-Packard DAD 1040M).

2.3. Oxygen-consumption rate

Oxygen-uptake experiments were performed to allow preliminary characterisation of the catabolic state of harvested cells, before performing enzyme assays or RT-PCR analyses. 10-ml samples of culture were concentrated five-fold in 50 mM ice-cold phosphate buffer. 0.5 ml of this suspension was added to the reaction chamber of a previously calibrated biological oxygen monitor (Rank Brothers Ltd. oxygen electrode) together with 2.5 ml of air-saturated Evans mineral salts media. Endogenous oxygen consumption was recorded for 5 min, after which the substrate of interest was added (10 μl of 10 mM PAA or a water-saturated styrene solution). Oxygen-consumption rates were corrected for endogenous uptake. All readings were carried out in triplicate and the averages are presented in Table 2.

2.4. Enzyme assays

SMO activities were measured via the production of indigo from indole, as previously described [6]. PAAK activity was assessed using a modified form of the assay employed by Martinez-Blanco et al. [7]. The need for crude cell-free extracts was eliminated by permeabilising cells with the non-ionic detergent Triton X-100 [8,14]. Washed cell suspensions were resuspended in 0.05% (v/v) Triton X-100 in 50 mM phosphate buffer and stored at −20°C for a minimum of 20 h [9]. The assay was then performed as according to Martinez-Blanco [7], with the permeabilised cells.

2.5. Phenylacetyl-CoA ester determination by high-performance liquid chromatography (HPLC)

Samples were taken from the assay and diluted 1:1 with methanol and centrifuged at 15 000 rpm for 30 min. The supernatant from the methanol-treated samples was frozen awaiting analysis by HPLC. Samples were analysed using a Nucleosil 100 HD C18 125×3 mm and a Hewlett-Packard HP1050 Ti HPLC coupled diode array detector (Hewlett-Packard DAD 1040M). Isocratic elution with 80% 20 mM phosphate buffer, pH 4.5, 9% methanol and 7% acetonitrile was performed at a flow rate of 0.5 ml min^{-1}. PAA and PACoA had retention times of 7.3 and 33.9 min respectively.

Table 1
Carbon source concentrations in the feed and chemostat, with associated culture OD_{540}, under various continuous culture conditions

Condition and carbon source	Concentration of (mM)		OD_{540}
	Substrate in feed	Residual PAA in chemostat	
C-lim			
PAA	5	0	0.7
PAA+succinate	5, 10	2.0	0.9
N-lim			
PAA	10	3.91	0.42
PAA+succinate	10, 20	8.33	0.57
S-lim			
PAA	10	4.14	0.35
PAA+succinate	10, 20	8.77	0.46
P-lim			
PAA	10	7.33	0.28
PAA+succinate	10, 20	9.10	0.45

2.6. RT-PCR

Total RNA was isolated from cells harvested under each of the limiting conditions according to Ausubel et al. [10] and concentrations were established using a GeneQuant II RNA/DNA calculator (Pharmacia Biotech). For each sample, 1 μg was reverse transcribed with 1 μl 10 mM Random Primer, 2 μl bovine serum albumin (1 mg ml^{-1}), 4 μl 5× buffer (Promega), 40 U RNasin (Promega) and 200 U MMLV-RT (Promega). Reactions were made up to 20 μl with diethylpyrocarbonate-treated demineralised water and incubated for 1 h at 37°C to generate cDNA. 2 μl of the RT reaction was then used as a template for subsequent PCR. PCR amplification of *styS*, *styR*, *styA* and *paaK* from cDNA was achieved using the specific primers previously described [6]. The number of amplification cycles used was optimised to avoid reaching a point at which band intensities, representing differing gene expression levels within cells, would be misleading due to a plateau of amplification having been achieved. Qualitative comparison of PCR product intensities was performed by densitometry with the GDS 8000 gel documentation system (UVP) and GelWorks 1D Intermediate analysis software.

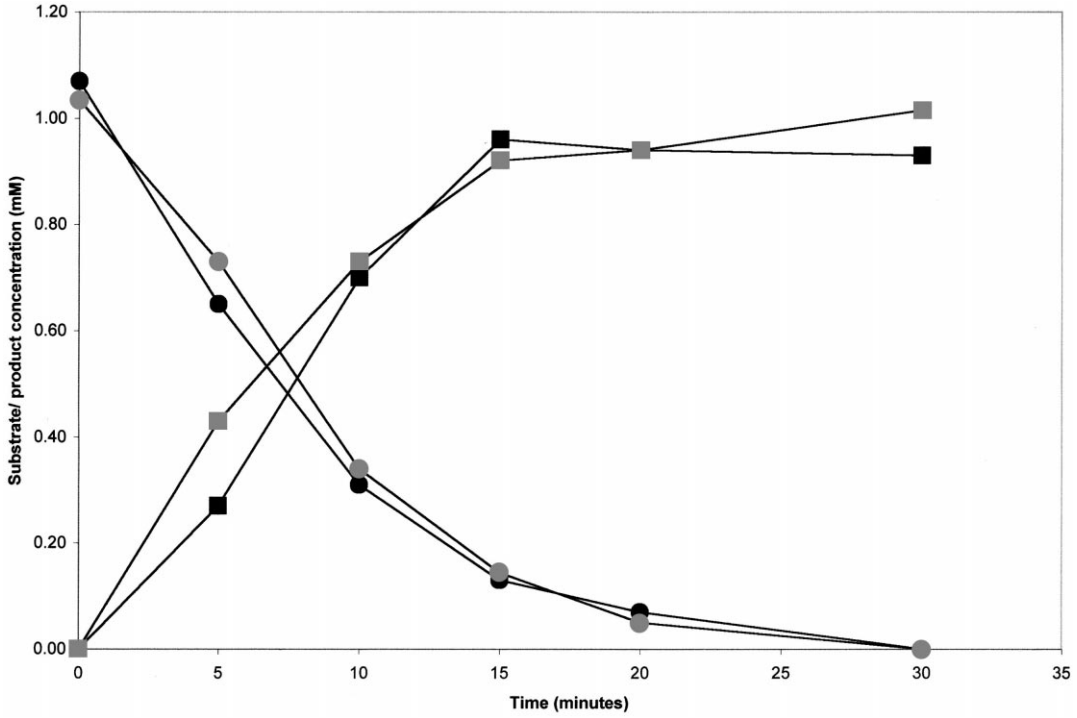

Fig. 1. Rates of PAA consumption and PACoA formation in permeabilised cells and cell free extracts. PAA consumption in permeabilised cells (grey circles) and cell-free extracts (black circles). PACoA formation in permeabilised cells (grey squares) and cell-free extracts (black squares).

Table 2
Oxygen consumption rates of washed cell suspensions of *P. putida* CA-3 grown on PAA under a variety of nutrient-limiting conditions

Substrate	Growth conditions			
	C-lim	C-lim, succinate	P-lim	P-lim, succinate
Styrene	85.5 ± 17.3	14.2 ± 1.4	ND	ND
PAA	300 + 22.1	110 ± 8.43	94 ± 5.0	ND

Uptake rates measured as nmol O_2 consumed min^{-1} mg cdw^{-1}. ND, below detectable levels.

3. Results and discussion

3.1. Growth monitoring

Once steady-state growth had been established, under each of the continuous culturing conditions, measurements of biomass (OD_{540}), pH and substrate concentrations in the chemostat were taken daily. The data generated is presented in Table 1 (the pH readings were not included as they did not fluctuate significantly during growth under the nutrient-limiting conditions investigated).

3.2. Validation of PACoA ligase assay with permeabilised cells

A PACoA ligase assay that combines cell lysis using a non-ionic detergent and a colourimetric assay in the same detergent solution was developed and validated by (a) correlating the appearance of a red colour in colourimetric assays to the appearance of PACoA in HPLC assays, and (b) comparing the rate of PAA depletion and PACoA formation using tritonated whole cells and cell-free extracts of chemostat grown *P. putida* CA-3 (PAA 15mM). The initial rate of PAA consumption was 15.2 ± 1.6 μmol min^{-1} g cell dry weight $(cdw)^{-1}$ for the cell-free extract and 13.9 ± 1.7 μmol min^{-1} g cdw^{-1} for tritonated cells of *P. putida* CA-3 respectively (Fig. 1). The rate of PACoA formation was 14.0 ± 3.2 and 13.6 ± 2.6 μmol min^{-1} g cdw^{-1} for cell-free extracts and tritonated cells respectively. The formation of a red colour corresponded to PACoA formation in HPLC assays and coincided with PA consumption for both cell-free extract and tritonated cells (data not shown).

3.3. C-lim

We have previously reported that the presence of PAA in batch cultures of *P. putida* CA-3 caused repression of *styA* gene transcription via complete inhibition of *stySR* expression [6]. In this study, however, it was observed that growth on limiting concentrations of the lower pathway substrate, PAA, resulted in the ability of washed whole cells to oxidise both PAA and styrene, (Table 2, column 1). The lower pathway PACoA ligase activity under these conditions was 48 nmol min^{-1} mg cdw^{-1}, which is 17-fold higher than that observed in PAA grown batch cultures of CA-3 (2.8 nmol min^{-1} mg cdw^{-1}) (Table 3) [6]. Analysis of total RNA revealed that this activity coincided with high levels of *paaK* gene expression (Fig. 3B, lane 2). Furthermore, significant transcription of *styS*, the histidine kinase sensor of the upper pathway positive regulatory apparatus was also observed, with concomitant induction of *styA* gene expression (Fig. 2, lane 2, Fig. 3A, lane 2). These data support preliminary observations made with whole-cell oxygen-consumption rates and activities of two other upper pathway enzymes for *P. putida* CA-3, grown under styrene and PAA carbon-limiting conditions [5]. Our observations suggest that, in this strain, the presence of limiting concentrations of the lower pathway substrate PAA induced expression of both the lower catabolic route and the upper pathway degradation enzymes responsible for PAA formation from styrene. These events were coordinated at the level of gene transcription. Enhanced substrate catabolism under conditions of C-lim is well established, however, bacterial responses to these conditions vary. According to Harder and Dijkhuizen [1], microorganisms may overcome low concentrations of carbon by either (a) increased uptake and intracellular accumulation of the substrate, or (b) enhanced initial metabolism of intracellular substrate. Our observations of high-level *paaK* gene transcription under PAA limitation, (Fig. 3B, lane 2), together with increased levels of PACoA ligase activity when compared with batch culture findings (Table 3, column 1) [6], supports the latter mechanism, with enhanced initial metabolism of PAA being facilitated by increased production of the initial substrate-activating enzyme, PACoA ligase. Similar studies on the TOL plasmid pWWO in *P. putida* have also shown that car-

Table 3
Specific enzyme activities of *P. putida* CA-3 grown on PAA under conditions of nutrient limitation and catabolite repression

Enzyme	Continuous culture conditions							
	C-lim	C-lim, succinate	N-lim	N-lim, succinate	S-lim	S-lim, succinate	P-lim	P-lim, succinate
SMO	2.2 ± 0.04	0.9 ± 0.01	ND	ND	ND	ND	ND	ND
PACoA	48 ± 3.5	15 ± 2.94	4.13 ± 0	ND	2.1 ± 0.18	ND	1.93 ± 0.17	ND

ND, not detected. Specific enzyme activities are both measured in nmol min^{-1} mg cdw^{-1}.

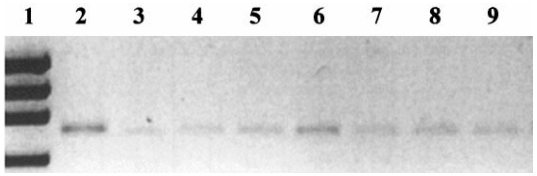

Fig. 2. RT-PCR analysis of total RNA for *sty*S expression in PAA-grown cells under nutrient-limiting conditions. Lane 1 = ϕX174 DNA marker, 2 = PAA-C-lim, 3 = PAA-N-lim, 4 = PAA-S-lim, 5 = PAA-P-lim, 6 = PAA+succinate-C-lim, 7 = PAA+succinate-N-lim, 8 = PAA+succinate-S-lim, 9 = PAA+succinate-P-lim.

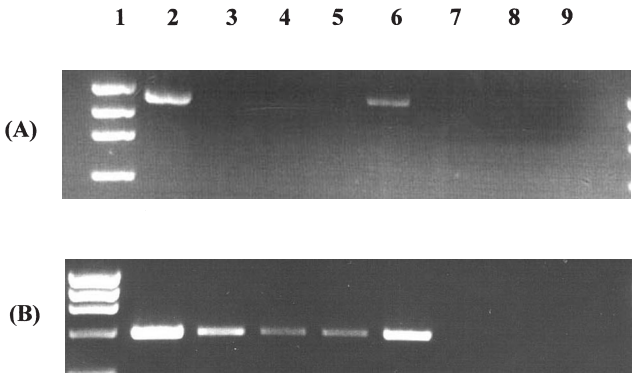

Fig. 3. RT-PCR analysis of total RNA from PAA-grown cells under nutrient-limiting conditions. A: *sty*A; B: *paak*. Lane 1 = ϕX174 DNA marker, 2 = PAA-C-lim, 3 = PAA-N-lim, 4 = PAA-S-lim, 5 = PAA-P-lim, 6 = PAA+succinate-C-lim, 7 = PAA+succinate-N-lim, 8 = PAA+succinate-S-lim, 9 = PAA+succinate-P-lim.

bon-limiting concentrations of pathway substrate resulted in elevated transcription of the toluene degradation upper and lower pathway genes [2,3,8].

3.4. Inorganic nutrient limitation

The introduction of inorganic nutrient limitation (N-, S-, P-lim) into cultures growing on non-limiting PAA caused a 10–20-fold decrease in PACoA ligase activities, compared with PAA-limiting growth conditions (Table 3, columns 3, 5 and 7). RT-PCR analysis of cells grown under these conditions revealed that this reduced lower pathway activity coincided with diminished transcription of the *paaK* gene (Fig. 3B, lanes 3–5). It was observed that repression of *paaK* was more stringent when sulfate or phosphate was limiting, and this corresponded with lower PACoA ligase activities than those recorded under N-lim, (Table 3, columns 3, 5 and 7). While expression of *styS* was observed under each of the anabolic limitations, levels appeared insufficient to subsequently induce transcription of *styA* (Fig. 2, lanes 3–5, Fig. 3A, lanes 3–5). These observations support the view of Magasanik [12] that any compound which can serve efficiently as a source of intermediary metabolites, and of energy, may reduce the rate of formation of catabolite-repression-sensitive enzymes. Thus, inorganic nutrient limitation gives rise to lower biomass yields, which result in an increase in residual PAA in the chemostat media (Table 1). Paradoxically, the cellular response to this excess substrate is to depress catabolism, assumedly until growth-supporting nutrient conditions are restored. The purpose of this repression may be to prevent the intracellular build up of toxic intermediates while cell growth is limited by the disruption of anabolic processes. It should be noted also that while both S- and P-lim resulted in almost identical PACoA ligase activities, the accumulation of residual PAA in the P-lim chemostat media was almost twice that recorded for S-lim (Table 1). It has been demonstrated previously that P-lim caused a change in the lipid composition of the outer membrane in a marine strain of *Pseudomonas fluorescens*, with lipids containing phosphatidyl-glycerol, phosphatidyl-ethanolamine and diphosphatidyl-glycerol being replaced by those containing ornithine [13]. The observed increase in residual PAA in the chemostat, when *P. putida* CA-3 was cultured under P-lim, may therefore reflect a reduced PAA-uptake efficiency due to an altered outer membrane composition. Interestingly, it was observed that in batch-grown cultures of *P. putida* U, 80% of radiolabelled PAA, actively transported into the cell, was unmodified [11]. This would suggest that CoA activation, and not substrate uptake, represents the rate-limiting step in the initial catabolism of PAA in *P. putida* U, under non-limiting conditions. In *P. putida* CA-3 inorganic nutrient limitation would appear to decrease both the level of PAA uptake into the cell, the expression of *paaK* and, consequently, the PACoA ligase enzyme activity responsible for its metabolism.

3.5. Catabolite repression

When *P. putida* CA-3 was grown in the presence of limiting concentrations of both PAA and succinate, a residual PAA concentration of 2 mM was detected in the chemostat media (Table 1). PAA oxygen-consumption rates of washed whole cells were approximately three-fold lower than under carbon-limiting growth with PAA alone (Table 2, columns 1 and 2), and this corresponded with a marked reduction in detectable PACoA ligase activity from 48 to 15 nmol min^{-1} mg cdw^{-1} (Table 3, columns 1 and 2). Gel densitometric comparisons of RT-PCR products generated suggested an almost two-fold decrease in *paaK* gene-expression levels following introduction of the primary carbon source, succinate, (Fig. 3B, lanes 2 and 6). These effects were reflected also in reduced transcription of *styS* and *styA*, (Fig. 2, lanes 2 and 6, Fig. 3A, lanes 2 and 6). These data suggested that a limiting concentration of succinate effected partial catabolite repression over the *sty* operon, via reduced transcription of both regulatory and structural genes.

Previous PAA oxygen-consumption rate analysis of *P. putida* CA-3 cells grown on non-limiting concentrations of PAA and succinate, in the presence of N- and S-lim, suggested enhanced catabolite-repressing effects of succinate,

on lower pathway activity, due to inorganic nutrient limitation [5]. In the present study it was observed that N-, S- and also P-limiting conditions resulted in an inability to detect PACoA ligase activity, when PAA and succinate were both present in the media (Table 3, columns 4, 6 and 8). RT-PCR analysis of total cellular RNA isolated under these conditions revealed that the loss of detectable PACoA ligase activity was concurrent with complete inhibition of *paaK* gene transcription (Fig. 3B, lanes 7–9), and was unlikely, therefore, to involve any post-translational enzyme regulatory mechanism. It should also be noted that while it was still possible to detect *styS* mRNA transcripts under these conditions, at levels comparable with those observed under inorganic nutrient limitation with PAA alone, there was no expression of *styA* (Fig. 2, lanes 7–9, Fig. 3A, lanes 7–9). These findings are supported by similar observations in *P. putida* (pWWO) where growth on 10–15 mM succinate, under limiting S and P concentrations resulted in a 98% loss of upper pathway BADH activity in response to the inducer *o*-xylene, compared with growth on limiting succinate alone [2,3].

It is clear from our observations that regulation of the styrene-degradation pathways, under continuous culture conditions, is achieved through induction and repression of the *sty* operon genes. These findings correspond with the currently emerging view of bacterial adaptation to nutrient limitation, as proposed by Ferenci, where it is believed that growth with suboptimal nutrient levels elicits adaptations not observed with either starving (resting) or unstressed bacteria. This group propose that nutrient limitation results in patterns of gene expression which optimise the scavenging capabilities of the organism through novel control mechanisms [14]. Increased upper and lower pathway gene transcription under C-lim, with only the lower pathway carbon substrate, PAA, present in the media, together with the similar effects elicited by all of the different anabolic limitations (N, S and P) and their involvement in catabolite repression by succinate, suggest the potential involvement of global, as well as specific, cellular regulatory processes. A variety of global, bacterial regulatory factors have been identified including ppGpp, acetyl phosphate, ATP levels and the DNA-binding proteins H-NS (the DNA binding domain of the H1 protein), and integration host factor. However, it is reported that their concentrations under nutrient-limiting growth conditions are poorly understood [14]. Regardless of the mechanisms involved, transcriptional repression of the styrene degradative pathway by nutrient-limiting conditions, has obvious implications for the application of this strain's catabolic potential in environmental bioremediation strategies. Our results demonstrate that inorganic limitation, which is a common feature of soil and aquatic environments, and in particular P-lim, might ultimately lead to accumulation of the xenobiotic through (a) depression of the catabolic ability, and (b) inducing stringent catabolite repression in the presence of primary carbon sources.

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