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Use of Starvation Promoters To Limit Growth and Select for Trichloroethylene and Phenol Transformation Activity in Recombinant *Escherichia coli*

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The expression of much useful bacterial activity is facilitated by rapid growth. This coupling can create problems in bacterial fermentations and in situ bioremediation. In the latter process, for example, it necessitates addition of large amounts of nutrients to contaminated environments, such as aquifers. This approach, termed biostimulation, can be technically difficult. Moreover, the resulting in situ bacterial biomass production can have undesirable consequences. In an attempt to minimize coupling between expression of biodegradative activity and growth, we used *Escherichia coli* starvation promoters to control toluene monooxygenase synthesis. This enzyme complex can degrade the environmental contaminants trichloroethylene (TCE) and phenol. Totally starving cell suspensions of such strains degraded phenol and TCE. Furthermore, rapid conversions occurred in the postexponential batch or very slow growth (dilution) rate chemostat cultures, and the nutrient demand and biomass formation for transforming a given amount of TCE or phenol were reduced by 60 to 90%. Strong starvation promoters have recently been cloned and characterized in environmentally relevant bacteria like *Pseudomonas* species; thus, starvation promoter-driven degradative systems can now be constructed in such bacteria and tested for in situ efficacy.

Except for the genes involved in secondary metabolite production, expression of most other bacterial genes is facilitated by rapid growth. However, many applied processes would be improved if certain bacterial metabolic activities could be expressed at significant levels during slow growth.

The process efficiency in industrial fermentations increases with increasing catalyst (biomass) concentration in bioreactors, and reactors attaining catalyst saturation have attracted wide interest (11). In such compact bioreactors, continued rapid cell growth can pose serious problems. The diffusivity of nutrients and products can be hampered, and in reactors with limited space, breach of the containment can result if cell growth is not restricted (11). Continued rapid growth is also a wasteful diversion of valuable substrate(s) from the desired end product to biomass. Decoupling of expression of desired metabolic activity from growth would therefore benefit process efficiency.

Such decoupling is also desirable in in situ bioremediation. Bacteria indigenous to polluted environments often possess the biochemical potential to degrade the contaminants yet bring about little remediation. This is generally because in natural environments a dearth of nutrients normally precludes significant growth, so that the required biochemical activities are only weakly expressed. Indeed, a major current technology of in situ bioremediation (termed biostimulation) relies on addition of nutrients to environments, such as aquifers, to induce the biodegradative activity and stimulate the metabolism of indigenous bacteria (8, 9, 25, 26, 28). For example, methane and oxygen have been supplied together to aquifers to biostimulate methylotrophs (26). The enzyme methane monooxygenase produced by these bacteria converts trichloroethylene (TCE) into organic acids that can be readily attacked and mineralized by other bacteria, thus affecting substantial removal of TCE. However, large amounts of nutrients must be added to achieve meaningful remediation, and considerable improvement will result if this need could be reduced and if biodegradative activity of slowly growing bacteria could be improved.

In a previous study (29), we utilized a special regulatory element of *Escherichia coli* to selectively express high levels of the reporter enzyme β-galactosidase in slowly growing cells. This regulatory element (the starvation promoter) was derived from a class of genes (the starvation genes) that are selectively switched on in a metabolically sluggish state. While most other types of gene expression are greatly attenuated, these genes exhibit a high level of expression in this state (1, 5, 14, 19, 20). Using a variety of approaches, we demonstrated that the starvation promoters permit induction and sustained production of β-galactosidase in cells rendered metabolically sluggish by nutrient deprivation (29).

The work reported here was undertaken to determine if expression of TCE and phenol-degrading activity can be improved in slowly growing cells so that more of the contaminant is removed per unit of biomass synthesized. For convenience, we refer to this ratio as conversion efficiency. We spliced the *tmoABCDE* operon to *E. coli* starvation promoters. Like methane monooxygenase, the polypeptides encoded by this gene cluster (toluene monooxygenase [TMO]) are believed to act as a mixed-function oxygenase in TCE and phenol degradation, in which NAD(P)H acts as the electron donor (31, 32). Reducing power is thus required for the degradation of these contaminants, and a factor in our choice of this reaction was to learn if cells rendered sluggish by a dearth of nutrients can adequately meet this demand. This is an important issue in the usefulness of starvation promoters. Phenol and TCE degradations are typical of other reactions of interest for applied processes in requiring reducing power and/or ATP expenditure.

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TABLE 1. Strains and plasmids used in this study

E. coli strain or plasmid	Relevant genotype	Reference(s) or source
Strains		
AMS66	K-12Δ*lacUV169 recA56 srl-300*::Tn*10*	1
AMS185	AMS66 bearing pMY402	This study
AMS186	AMS66 bearing pAMC202	This study
AMS187	AMS66 bearing pAMC203	This study
AMS188	AMS66 bearing pAMC201	This study
Plasmids		
pMY402	*lacI*^{q} $P_{tac\text{-}tmo}$	33
pAMC201	*tmo* (promoterless)	This study
pAMC200	*cstC-lacZ*	1, 12
pOF39	*groEL-lacZ*	15
pAMC202	*cstC-tmo*	This study
pAMC203	*groEL-tmo*	This study

Thus, apart from ensuring that the necessary enzyme(s) will be produced by the metabolically sluggish cells, it is also important to determine whether such cells can fuel energy-requiring reactions. The starvation promoter with which most of this work was carried out (P_{groEL}) is markedly induced upon starvation but is also expressed constitutively at significant levels during rapid growth. This enabled us to compare conversion efficiencies in rapidly growing cells with those in cells subjected to different kinds and degrees of starvation by using the same enzyme system. Preliminary reports of these findings have been presented (17, 21).

MATERIALS AND METHODS

Organisms and culture conditions. Our laboratory *E. coli* strain, strain AMS66 (27), was used as host for the plasmids employed in this study; the different strains and plasmids used are listed in Table 1. Cultures were grown in minimal medium plus glucose and were incubated with shaking at 37°C. Most experiments were carried out with M9 medium with the following composition (per liter): Na_2HPO_4, 6 g; K_2HPO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.246 g; and $CaCl_2 \cdot 2H_2O$, 15 mg; D-glucose was added at specified concentrations. To determine the effect of starvation for different nutrients, a non-phosphate buffer-containing medium (Tris-hydrochloride medium) was used, so as to maintain buffering capacity during phosphate starvation without phosphate in the medium, as described previously (5). The composition of this medium was as follows: 0.05 M Tris hydrochloride (pH 7.2), 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 μM $FeCl_3$, 10 mM Na_2SO_4, 0.1 M Na_2HPO_4 (pH 7.2), and 20 mM NH_4Cl. The glucose concentration was as specified. Either Na_2HPO_4, NH_4Cl, or glucose was omitted from the medium to obtain phosphate, nitrogen, or carbon starvation conditions, respectively.

Construction of plasmids with *tmo* operon under the control of a starvation promoter. The *tmo* gene complex codes for TMO, which can fortuitously degrade TCE and phenol into compounds which can be completely mineralized in a natural mixed population (4, 33); the physiological role of this enzyme complex is to permit utilization of toluene for growth. Winter et al. (33) kindly supplied their plasmid pMY402. In this plasmid, a DNA fragment from *Pseudomonas mendocina* KR-1, bearing the *tmo* structural gene cluster (34), is controlled by the *tac* promoter (P_{tac}); *E. coli* strains bearing pMY402 degraded TCE during rapid exponential growth when P_{tac} was induced by isopropyl-β-D-thiogalactoside (IPTG), as reported previously (33).

A 4.5-kb DNA fragment, bearing the *tmo* operon, was removed from pMY402 (*Eco*RI and *Ppu*MI digestions) and was inserted in place of the corresponding (ca. 1.6-kb) restriction fragment in pBR322, generating pAMC201 (Fig. 1; Table 1). A 1.6-kb *Eco*RI-*Bgl*II or a 300-bp *Eco*RI-*Sau*3A fragment from appropriate plasmids (pAMC200 or pOF39), bearing either the *cstC* (1, 12) or *groEL* (5, 15) starvation promoter (P_{cstC} or P_{groEL}, respectively) was inserted into pAMC201 by using the appropriate polylinker sites just upstream of the *tmo* operon (the *Bam*HI site of pAMC201 and the *Bgl*II sites of *cstC* [Fig. 1] share a compatible overlap). The resulting plasmids, pAMC202 and pAMC203, contain *tmo* under the regulation of P_{cstC} or P_{groEL}, respectively. The desired transformant colonies were selected by their blue color on Luria-Bertani agar plates, which is produced by the fortuitous conversion of indole into indigo by TMO.

Growth and bioconversion assays. For TCE, growth and bioconversion assays were done by a modified version of the method of Little et al. (18). Cultures were grown in 250-ml septum bottles containing 100 ml of medium. These were dosed with enough unlabeled and labeled [1,2-^{14}C]TCE to give the stated initial concentrations at a specific activity of ca. 10,000 cpm/nmol of TCE. On the basis of established practice (18, 24, 33), the final concentration was calculated on the assumption that all the TCE remained in the liquid phase. A_{660} (to measure growth) and residual TCE concentration were determined at appropriate intervals.

To determine phenol bioconversion, cells were grown in 250-ml Erlenmeyer flasks capped with cotton plugs; phenol was added to different cultures to give the indicated initial concentrations. There was no measurable loss of phenol by evaporation for the duration of the experiments, as determined with AMS66 control cultures.

Chemostat experiments. Chemostat experiments were performed as described previously (6, 16). The bacteria were grown at 37°C in a New Brunswick C-30 Bioflo chemostat with a working volume of 550 ml with 0.1% glucose–M9 as the inflow medium. The aeration rate was 0.5 liter/min, and agitation was maintained at 250 rpm. The pH remained at 7.0 throughout the cultivation. Carbon-limiting conditions were obtained by using glucose limitation. At least five volume changes were allowed before the cultures were considered to be in steady state; the culture density remained constant during this time. Mutant selection was warded against by starting each chemostat run with a fresh inoculum (6).

Bioconversion assays in starving cell suspensions. Mid-log-phase glucose-Tris-hydrochloride medium-grown cells were harvested in a prewarmed centrifuge rotor. They were suspended to a cell density of 0.42 mg of protein per ml in the same medium lacking either carbon, nitrogen, or phosphorus. For phenol experiments, the cultures were incubated in Erlenmeyer flasks aerobically with shaking and the phenol concentration was assayed at appropriate intervals. TCE degradation experiments were conducted in glass vials capped with Teflon-lined silicone rubber septa to which cell suspension and [^{14}C]TCE were added. At periodic intervals, 1-ml aliquots were withdrawn for TCE assay.

Analytical methods. The residual TCE concentration was determined by procedures previously used by us and others (18, 24, 33). Pure cultures of recombinant *E. coli* with cloned TMO convert TCE mainly into water-soluble organic acids and CO_2, with little incorporation into cell material (24, 33). Thus, to determine the residual (untransformed) TCE concentration, 1-ml aliquots were withdrawn from cultures into syringes containing 1 ml of hexane. Unreacted TCE

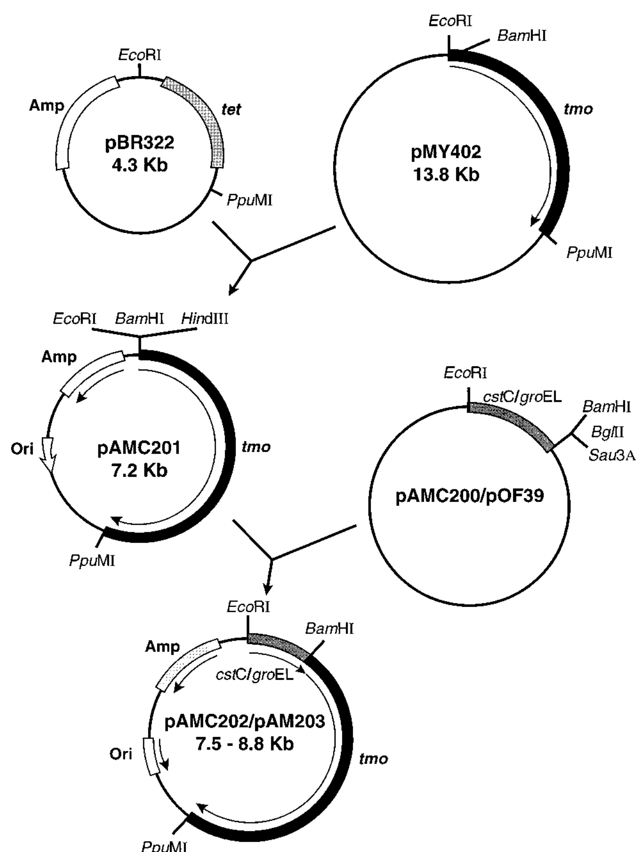

FIG. 1. Construction of plasmids pAMC201 (with promoterless *tmo* gene complex), pAMC202 ($P_{cstC\text{-}tmo}$), and pAMC203 ($P_{groEL\text{-}tmo}$). See the text for details.

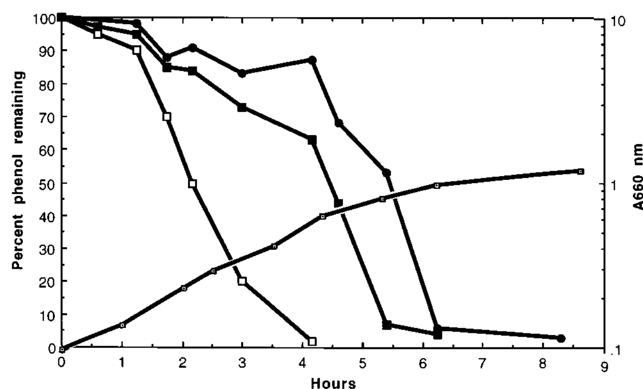

FIG. 2. Degradation of phenol by AMS187 cultures in exponential and postexponential phases in 0.1% glucose–M9 medium. The cultures contained 100 μM (□), 400 μM (■), or 700 μM (●) initial phenol concentration. The growth pattern (A_{660}) with and without the above phenol concentrations is also shown (⊡); the deviation in individual growth data points was never more than 5%. The concentrations at the end of the exponential phase were 0, 300, and 600 μM, respectively.

becomes trapped in the hexane layer and was counted in a Beckman liquid scintillation counter. Phenol was measured by a colorimetric antipyrene dye method (4). A linear relationship was found between A_{550} and phenol concentration between 0 and 1,000 μM.

Biomass in all experiments was monitored by A_{660} measurement, and the optical density units were converted to protein or dry weight values by use of appropriate standard curves (27). All rate determinations were replicated in at least two independent runs; the variation was within 10%.

RESULTS

Phenol degradation during exponential and postexponential phases by AMS187. The generation time of AMS187 (in which *tmo* expression is controlled by P_{groEL} [Table 1]) in 0.1% glucose–M9 medium was ca. 100 min (Fig. 2); the exponential phase under these conditions lasted for 4.5 h, at which time the culture contained a biomass of 0.32 mg of protein per liter. During the subsequent postexponential phase, growth occurred at a decelerating rate and then ceased; when growth ceased, the culture contained a total biomass of 0.43 mg of protein per liter. We have shown previously that the end of the exponential phase under these conditions coincides with the exhaustion of glucose from the medium, and the subsequent decelerating growth is fueled by the acetate that accumulates in the medium (29). Inclusion of phenol at concentrations ranging between 100 and 700 μM had no effect on this growth pattern.

The degradation kinetics of phenol at different concentrations are plotted in Fig. 2 as a percentage of the initial concentration of phenol remaining in the medium at different times so as to fit all the data on one graph. In the exponential phase, the amount of phenol converted by the end of the phase was the same (ca. 100 μM), regardless of the initial concentration of phenol (100, 400, or 700 μM). Consequently, at the onset of the postexponential phase, the last two cultures still contained 300 and 600 μM phenol, respectively (Fig. 2). In both the cultures, phenol was converted at ca. 674 μmol/h/mg of cell protein in the postexponential phase, and virtually complete conversion occurred at ca. 1 and 2 h, respectively, after the onset of this phase in the two cultures.

Not only were the conversion rates higher in the postexponential phase but also more conversion occurred per unit of biomass synthesized in this phase. At all three phenol concentrations, the conversion efficiency in the exponential phase was (100/0.32) 310 μmol of phenol converted per mg of cell protein synthesized. In contrast, in the postexponential phase, this efficiency in the two cultures was (295/0.11) 2,682 or (595/0.11) 5,409 μmol of phenol converted per mg of protein synthesized.

TCE degradation by AMS187. In contrast to phenol, TCE inhibited both the growth rate and yield of strain AMS187 (Fig. 3). At 10 μM TCE, the growth rate decreased to an 8-h generation time; at higher concentrations, there was a lag of 4 to 6 h and the growth was even slower and ceased at some 60% of the yield attained in control cultures (Fig. 2).

At 10 μM TCE, degradation proceeded during exponential growth, by the end of which some 65% of the initial TCE had been degraded; the conversion efficiency was 18.6 μmol of TCE per mg of cell protein synthesized (Fig. 3). No further degradation was observed in the postexponential phase, although 3.5 μM TCE remained in the medium. Similarly, at higher TCE concentrations, little or no degradation occurred in the postexponential phase.

In nearly all biological systems used in TCE bioremediation, a residual of TCE persists (28, 33), which may represent the eventual poisoning of the cells with intermediates of TCE breakdown, like the TCE epoxide (18, 30), and this may have been why no degradation was observed in late growth phase in the above experiments. We therefore investigated TCE degradation by postexponential-phase cells under conditions where they would be only minimally exposed to TCE degradation products before being tested for TCE transformation.

This condition could be fulfilled by adding TCE to cultures as they entered the postexponential phase, but this would have necessitated exact determination of the time point of entry into this phase, which is technically difficult (29). Instead, we utilized strain AMS186, in which *tmo* expression is driven by the *cstC* promoter (Fig. 1; Table 1), which is expressed only minimally in exponential phase (at about 1/10 the level of the *groEL* promoter [5, 12]). Cells of strain AMS186 were inoculated in 0.05% glucose–M9 medium supplemented with 12 μM TCE, and growth and TCE disappearance were monitored. Only a small amount of TCE was transformed until the onset of the postexponential phase (Fig. 4), but rapid degradation occurred in this phase. The rate of conversion was 6.6 nmol/h/mg of protein, and the conversion efficiency was 350 μmol of TCE per mg of protein synthesized. A similar experiment was performed with strain AMS188; this strain bears the same plasmid as AMS187 except that the *tmo* operon in this plasmid is without a promoter (Table 1; Fig. 1). There was no conver-

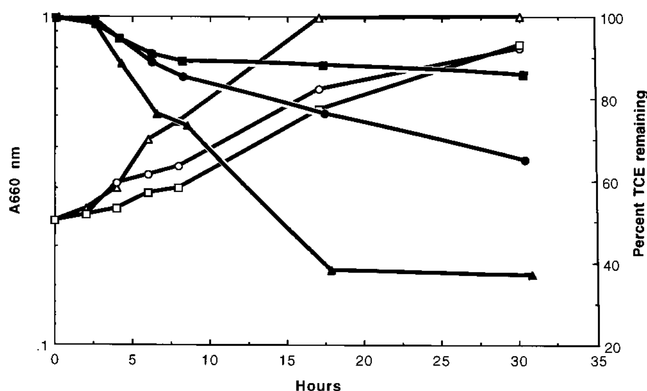

FIG. 3. Growth of (open symbols) of and TCE transformation by (solid symbols) strain AMS187 (bearing plasmid pAMC203 [$P_{groEL\text{-}tmo}$]) in the presence of 10 μM (△, ▲), 50 μM (○, ●), or 250 μM (□, ■) TCE. The M9 medium contained 0.1% glucose.

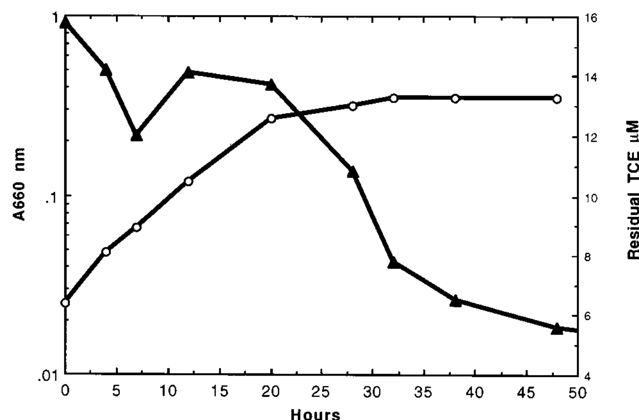

FIG. 4. Transformation of TCE by strain AMS186 ($P_{cstC\text{-}tmo}$) cultures during exponential and postexponential phases in 0.03% glucose–M9 medium. Symbols: ○, A_{660}; ▲, residual TCE.

sion of TCE during the exponential or postexponential phases (data not shown), confirming that the results in Fig. 4 were due to P_{cstC}-driven expression.

Transformation of phenol by chemostat-grown AMS187 cells. Both AMS187 and AMS186 carried out transformations at high rates with minimal biomass formation in the decelerating phase of growth in the above batch culture experiments (Fig. 2 and 4). Since in a batch culture a rapid succession of progressively falling growth and metabolic rates occurs in this phase, it was not possible to determine which rates were most conducive to the degradative activity expression. In chemostat cultures, however, the growth rate equals the dilution rate, and a whole range of growth and metabolic rates can be obtained in a time-independent fashion. Thus, a more precise determination can be made of the relationship between growth (metabolic) rates and the expression of transforming activity.

AMS187 was grown at a number of different dilution (growth) rates under glucose limitation. Aliquots were removed from steady-state cultures and analyzed for their phenol-degrading activity. Since the activity of interest was the degradation, we did not investigate whether the changes in this activity caused by growth rate were due to changes in the cellular TMO specific activity, availability of reducing power, or both.

The specific phenol-degrading activity showed a complex pattern in response to growth rate (Fig. 5). It decreased as the growth rate was reduced from the maximal (μ_{max} of 0.43 h^{-1} or a 1.6-h generation time) to 0.15 h^{-1} (4.6-h generation time). Further reduction in growth rate, however, led to an increase in degrading activity, so that at the very low growth rate of 0.05 h^{-1} (14-h generation time), this activity had gone up some 1.6-fold.

The amount of contaminant removed per unit of biomass synthesized was calculated from these data. For example, at a 14-h generation time, assuming a starting biomass equivalent to 1 mg of cell protein, there would be a net synthesis of 0.07 mg of cell protein per h. Given the phenol degradation rate at this growth rate (87 nmol of phenol removed per mg of cell protein per h [Fig. 5]), this means that 1,243 nmol of phenol will be degraded per mg of protein equivalent of biomass synthesized. At a 1.6-h generation time, the corresponding amount was only 330 nmol. Figure 5 shows that this decoupling from growth of TMO-mediated phenol removal increases progressively as the growth rate is reduced below 0.15 h^{-1}. In contrast, strain AMS185 ($P_{tac\text{-}tmo}$; in the presence of IPTG) was inactive in transforming phenol after growth at 0.05 h^{-1}.

Degradations by strain AMS187 during total starvation. Promoters like *groEL* and *cstC* are also induced during complete starvation such as that attained by suspending growing cells in a medium devoid of an essential nutrient. Under such conditions, any requirement for the missing nutrient in affecting a transformation must be met entirely from endogenous sources. It was of interest to determine how long phenol and TCE degradations could be sustained under such conditions, especially since these transformations require reducing power. As stated in Materials and Methods, exponentially growing cells of strain AMS187 were removed from the growth medium by centrifugation and were suspended in carbon, nitrogen, or phosphorus starvation medium.

Carbon-starved cultures reduced the TCE concentration rapidly in the first 60 min, and the degradation then slowed; some 35% of the TCE had been removed by the end of the experiment (Fig. 6A). Carbon-starved AMS187 also degraded phenol, and again the rate decreased with time and about 43 μM phenol was converted by the end of the experiment (Fig. 6B).

The nitrogen-starved cultures caused a more rapid disappearance of TCE and phenol from the medium than the carbon-starved cultures did (Fig. 6); the residual contaminant concentrations were also lower. Only phenol degradation was determined during phosphate starvation, and a rate intermediate between those for carbon and nitrogen starvation was found (data not shown). The initial specific rates of degradation under these conditions are presented in Table 2.

In all of the above starvation experiments, degradation by strain AMS185 was also determined. The expression of *tmo* in this strain is under the control of P_{tac}, which is induced strongly in growing cells when IPTG is added. Measurements with AMS185 served as a control of degradative activity that might be obtained under conditions of complete starvation with a nonstarvation promoter. Little degradation of TCE or phenol was observed when this strain (plus IPTG) was used under carbon starvation; somewhat more degradation was seen under nitrogen starvation, but it was much less than that effected by strain AMS187 (Fig. 6). The $P_{tac\text{-}tmo}$ construct present in this strain effectively degrades TCE in rapidly growing *E. coli* cells, as shown by Winter et al. (33) and confirmed by us in this study.

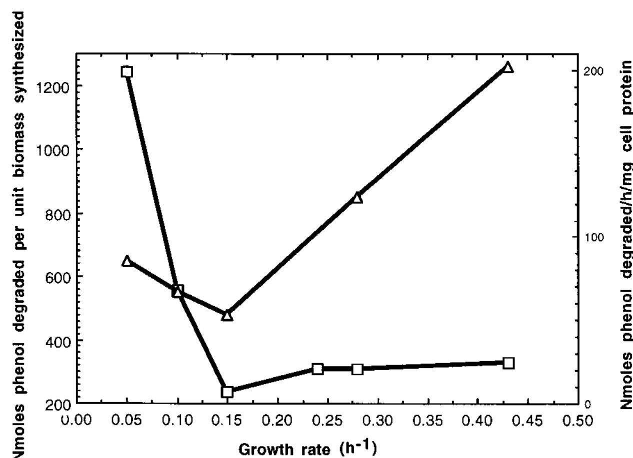

FIG. 5. Effect of growth (dilution) rate in chemostat-grown cells of strain AMS187 on the specific phenol degradation rate (△; expressed as nanomoles of phenol degraded per hour per milligram of cell protein) and the amount of phenol converted per unit of biomass synthesized (□). See the text for further discussion.

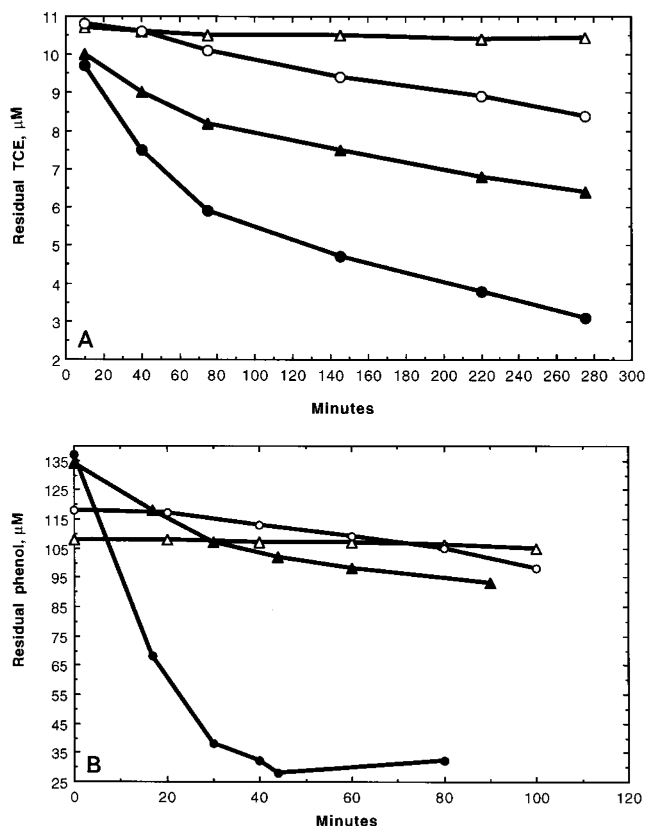

FIG. 6. TCE (A) and phenol (B) degradation by starving cell suspensions of AMS187 (solid symbols) or AMS185 ($P_{tac\text{-}tmo}$) (open symbols). Symbols: ▲, △, glucose starvation; ●, ○, nitrogen starvation. IPTG (1 mM) was added to the AMS185 suspensions. Cells were grown in 0.2% glucose–Tris-hydrochloride medium, harvested in mid-log phase, and resuspended in the same medium but lacking either carbon, nitrogen, or phosphorus.

DISCUSSION

There is abundant evidence that much useful microbial transforming activity is poorly expressed in metabolically sluggish cells, its expression being controlled by promoters requiring rapid growth. Indigenous bacteria possessing bioremediation capacities are ineffective in environmental restoration unless they are stimulated by the provision of large amounts of nutrients (4, 9, 28), and the ethanol-forming ability of yeast cells decreases precipitously when they are starved (10). Similarly, TCE degradation by methylotrophs in vitro (18) as well as in situ (28) virtually ceased upon discontinuation of the methane supply. Results presented here further show that P_{tac}, a powerful promoter in growing *E. coli* cells (33) and other organisms (2), is ineffective in driving TMO-mediated TCE or phenol degradation in starving cells.

This coupling of biotransforming effectiveness with rapid growth creates difficulties in both bioprocessing and bioremediation. These difficulties arise from the production of large amounts of biomass and the waste of nutrients that it entails. For instance (23), bioremediation of 1,000 gal (3,785 liters) of gasoline by natural bacterial populations would generate 7,000 lb (3,175 kg) of bacterial biomass and consume large amounts of various nutrients (e.g., 10,000 lb [4,536 kg] of oxygen). Provision of nutrients in such amounts is difficult; furthermore, the resulting biomass could clog subsurface pores, disrupting groundwater movement.

The central purpose of the work reported here was to determine if starvation promoters can selectively enrich expression of phenol- and TCE-degrading activity in metabolically sluggish bacteria so as to increase transforming activity per unit of biomass synthesized. We used totally starving cell suspensions, as well as batch and continuous cultures, to compare conversion efficiencies for high and low growth (metabolic) rates.

Totally starving cells of AMS187 transformed TCE and phenol, with higher rates occurring under nitrogen or phosphate starvation than under carbon starvation. This is presumably because reducing power is more abundantly available under the former starvation conditions. In all cases, however, the reactions rates were low and tended to level off with time. It is possible that availability of a balanced nutritional milieu is necessary for sustained expression of metabolic activity regardless of the nature of the promoter used.

Nonetheless, the use of starvation promoters made it feasible in both batch culture and continuous-culture systems to slow growth to a greater extent than the expression of the activity controlled by these promoters. For convenience, Table 2 includes all the conversion efficiencies and an estimate of the amount of biomass generated and (from yield data of this and previous [22] studies) glucose consumed, per millimole of contaminant transformed under various conditions. These data show that the use of a starvation-inducible promoter leads to a 4- to 17-fold enrichment of transforming activity in metabolically sluggish cells compared with rapidly growing cells, with the result that 60 to over 90% less biomass is formed and 60 to

TABLE 2. Removal rates and conversion efficiencies

Conditions	Rate (μmol/mg of protein/h)		Efficiency (μmol converted/mg of biomass synthesized)		Biomass (mg of protein) generated per mmol of contaminant degraded		Amt of glucose (g) required to transform 1 mmol of contaminant	
	Phenol	TCE	Phenol	TCE	Phenol	TCE	Phenol	TCE
Batch (exponential)	—[a]	—	310	18.6	3.22	54	26	436
Batch (postexponential)	674	6.6	2,682–5,409	350	0.37–0.19	2.8	3–1.5	22
Carbon starvation	0.15	0.004	∞	∞	ND[b]	ND	NA[c]	NA
Nitrogen starvation	1.2	0.012	∞	∞	ND	ND	NA	NA
Chemostat, carbon limitation (generation time, 1.6 h)	0.022	—	330	—	3.03	—	24	—
Chemostat, carbon limitation (generation time, 14 h)	0.009	—	1,243	—	0.8	—	6.5	—

[a] —, not determined.
[b] ND, not detected.
[c] NA, not applicable.

over 90% less glucose is consumed in affecting the transformation of a unit amount of TCE or phenol (Table 2). In a recent study (8), the conversion efficiency of TCE transformation by biostimulation of indigenous bacteria was found to be 524 and 929 mg of cell dry weight generated per mmol of TCE transformed in laboratory cultures and in situ, respectively. With our starvation promoter system and using the TCE degradation data in the decelerating phase (Table 2), the corresponding value for laboratory cultures if 5.6 mg of cell dry weight, i.e., less than 100-fold. This is consistent with the hypothesis that the use of starvation promoters could reduce the biomass generated in bioremediation and hence the need for biostimulation.

E. coli is not indigenous to contaminated environments, and therefore its recombinant strains cannot be considered for release into the environment for in situ bioremediation. However, powerful starvation promoters exist in *Pseudomonas* species (3) and have recently been cloned from *Pseudomonas putida* (13), and technology exists to integrate into its chromosome genes spliced to such promoters to obtain stable recombinant strains (7). Thus, the principle illustrated by the studies presented here deserves consideration for applicability to in situ bioremediation. Several questions would arise in this context. Can such recombinant *Pseudomonas* strains survive in situ and compete with the indigenous population? Would it be necessary to add them in large enough numbers from the outset so that appreciable bioremediation could occur, or would it be possible to grow them to the required density in situ? What level of biostimulation would be required to attain the desired bioremediation, and how much biomass will be produced—how would these numbers compare with laboratory findings? Would these recombinant strains pose some unforeseen environmental hazard, and could they be eliminated from the environment if they did? None of these questions can be answered at present, and considerable research would be needed to address them. However, given the not inconsiderable problems of the current technology of in situ bioremediation, it would seem prudent to pursue these questions.

ACKNOWLEDGMENTS

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