Engineering *Pseudomonas putida* to minimize clogging during biostimulation

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Abstract

Bacterial bioremediation is a safe and inexpensive means of decontaminating environmental pollution. However, low nutrient concentrations at polluted sites frequently limit bioremediating activity. Biostimulation by exogenous application of nutrients does not always promote effective transformation of pollutants, and the high levels of biomass that result can cause clogging around feeding ports, further constraining this strategy. To overcome these limitations we propose the application of starvation promoters to selectively express genes that are useful in bioremediation at maximal levels in nutrient-limited cells. Here we show that placing toluene monoxygenase (TMO) genes under control of the Pstarv1 starvation promoter in *Pseudomonas putida* MK1 brought about an 8-fold increase in the rate of phenol degradation by stationary-phase cells over exponentially growing cells. Under nutrient-limiting conditions these cells were also able to transform trichloroethylene with a conversion efficiency approximately 64-fold greater than unmodified cells, which only express appreciable levels of TMO in exponential phase. We also mapped and characterized the native promoter of *chrR*, a *P. putida* gene encoding a chromate-reducing enzyme, and show that it is likely to be under the transcriptional control of sigma 32, a heat shock and starvation regulated sigma factor. Consistent with this observation, *P. putida* cells grown at different dilution rates in a chemostat exhibited maximal chromate-reducing activity at low growth rates. These results are promising for maximizing the expression of chrR under field conditions for chromate bioremediation.
Keywords: bioremediation, biostimulation, phenol, trichloroethylene, hexavalent chromium, toluene monoxygenase, chromate reductase, starvation promoter.

1 Introduction

Environmental pollution is a serious problem world-wide. While current remedial solutions such as excavation, incineration, pump and treat, and entombment, are viable approaches, they nonetheless have considerable disadvantages, such as exposure of workers to potential hazards, increased inputs to waste repositories, and high expense. Bioremediation has the advantage of being able to restore damaged environments at lower cost, more rapidly and with lower human risk. There are, however, several technical barriers to the effective application of this technology to environmental cleanup. In the first place, most of the environmental pollutants that are harmful to humans are toxic also to the bacteria that can remediate them, greatly impeding their beneficial activity. Second, polluted sites generally possess a mixture of different toxic compounds. At the Department of Energy waste sites in the United States, for example, which span over 2,800 square miles, heavy metals, radionuclides, complexing agents, chlorinated and fuel hydrocarbons, organic solvents, parachlorobiphenyls (PCEs), and ketones co-exist in different combinations at different sites; Riley and Zachara [1]. This mixed waste can be lethal to indigenous microorganisms required to detoxify individual contaminants: solvents can kill metal-resistant microbes; metals, the solvent-resistant ones; and radionuclides, the radiation-sensitive bacteria.

Another hurdle facing effective bioremediation by bacteria is that most polluted sites possess low nutrient concentrations, making microbial growth so slow that their remediating activities cannot be effectively expressed. A solution to this problem is provided by the process of "biostimulation," in which nutrients are added to the environment to stimulate the growth of indigenous bacteria, thus enhancing their transforming activity. However, biostimulation of natural populations can result in large bacterial biomass formation and nutrient consumption. Many reactions useful in bioremediation are fortuitous ("co-metabolic") processes from which the remediating bacterium derives no benefit. Only small proportions of the available nutrients (typically no more than 0.005 - 1.6% of the available electrons) are consumed on these processes since bacteria would rather grow than remediate. Estimates are that dehalogenation of 16.3 kg of PCE (a typical amount per m^3 of a contaminated soil) would require 970 kg of lactate and result in 140 kg of bacterial biomass; Bouwer [2]. Such biomass formation can cause clogging around the feeding port, confining effective remediation to a narrow zone; McCarty and Semprini [3].

For all these reasons, there is growing recognition that the powerful tools of genetic and molecular engineering can provide a solution to these complex problems. These approaches can likely permit generation of bacteria that can survive and remediate multiple contaminants, and are able to function under the low nutrient conditions of the polluted sites. However, such approaches have so far been sparingly applied to addressing bioremediation problems, and their potential for bioremediation remains largely to be demonstrated. This demonstration will go a long way in mitigating public concerns about the use of "artificial" life forms in environmental cleanup, especially when information is

also provided on the excellent means that are available for controlling the spread of engineered microbes, should the need arise; Lewis [4].

We have been using molecular approaches to address both of the problems mentioned above. Thus, we have identified and cloned genes encoding bacterial enzymes that detoxify chromate in a way that is least harmful to the remediating bacterium. Three such "safe" enzymes have been characterized, two in *Escherichia coli* (YieF and NfsA), and one in *Pseudomonas putida* (ChrR); Ackerley et al [5, 6]. We have shown that recombinant bacteria overproducing these types of enzymes exhibit decreased chromate toxicity making them more effective agents of chromate bioremediation; and are currently using DNA shuffling technology to increase the efficacy of these enzymes for chromate reduction and to amplify their range, so they can remediate additional contaminants.

For the clogging problem associated with biostimulation, we have made use of special regulatory elements of *Escherichia coli* to selectively express high levels of different enzymatic activities in metabolically sluggish cells; Kim et al [7]; Matin [8]. These regulatory elements (the starvation promoters) were derived from starvation genes, which are selectively switched on in slowly growing cells. While the expression of most other genes is greatly attenuated, starvation genes exhibit a high level of expression in this state; Blum et al [9]; Matin et al [10]; McCann et al [11]; Tunner et al [12]. Using a variety of approaches, we demonstrated that starvation promoters permit induction and sustained expression of desired enzyme activities in slowly growing cells, so that marked transformations can be achieved with much reduced biomass production; Tunner et al [12].

In a "proof-of-concept" study, we examined TCE and phenol degradation in recombinant *E. coli* strains. These strains contained plasmids in which the expression of the toluene monoxygenase enzyme complex (TMO) was controlled by starvation promoters; Matin et al [13]. TMO is encoded by the *tmoABCDEF* operon, and is a mixed function oxygenase cloned originally from *Pseudomonas mendocina*; Yen et al [14]. Its physiological role is to enable *P. mendocina* to use toluene as a growth substrate. But it can, co-metabolically, also degrade phenol and TCE to products that can be readily attacked and mineralized by other bacteria. Recombinant *E. coli* bearing starvation promoters (e.g., P*groEL* or P*cstC*; Kim et al [7]) spliced to the *tmo* genes degraded phenol and TCE with a high conversion efficiency (i.e. the amount of contaminant degraded per unit amount of biomass synthesized and growth substrate consumed). This efficiency was over a hundred-fold greater than of the natural populations; Matin et al [13].

Since *E. coli* is not indigenous to polluted environments, we have applied the same principle to constructing recombinant *P. putida* strains, which are almost universally present in polluted sites. Our previously characterized and cloned *P. putida* starvation promoter (P*starv1*; Kim et al [7]) was spliced to the *tmo* gene cluster. P*starv1* is expressed at a significant level during exponential growth, but is markedly induced in the post-exponential phase. We report here the TCE and phenol conversion efficiency and other characteristics of *P. putida* containing a P*starv1-tmo* construct. In addition, we describe characterization of the regulatory

elements that control the expression of the *chrR* gene in wild type *P. putida*. These findings will facilitate construction of a strain capable of expressing an improved safe chromate reductase in low-nutrient environments.

2 Materials and methods

2.1 Bacterial strains, plasmids, culture condition and chemicals

All strains and plasmids used in this study are listed in Table 1. Cells were grown in glucose-M9 medium. Cell growth was determined by measuring A_{660}; glucose by the Glucose [HK] 10 enzyme kit (Sigma); and total cell protein by the Bio-Rad DC protein assay kit. Indole and indigo were purchased from Sigma.

All plasmid manipulations were performed in *E. coli* DH5α or JM109. Plasmid transfer from *E. coli* to *Pseudomonas* strains was accomplished by triparental mating in the presence of the helper plasmid pRK600, as described previously; Kim et al [7]. The donor, recipient and helper cells (1:2:1, respectively) were collected on a 0.2 μm syringe filter (Nalgene). The filters were placed on LB agar plates and after allowing growth for 8 h at 30 °C , the mating mixture was suspended in 0.1% phosphate buffer and streaked on *Pseudomonas* Isolation Agar (Difco). Carbenicillin was used to select the *Pseudomonas* transformants.

Table 1: Strains and plasmids.

Bacterial strain or plasmid	Relevant characteristics	Reference
Strains		
E. coli		
DH5α	*hsdR17*($r_K^-m_K^-$) *supE44 thi-1 recA1 relA1 gryA (lacIZYA-argF)*	NEB
JM109	JM107 *recA1*	NEB
P. putida		
MK1	Derivative of ATCC12633: Rif^r	7
AMS1001	MK1 with pAM103	This study
AMS1002	MK1 with pAM104	This study
P. mendocina		
KR1	The strain from which *tmoABCDEF* was cloned	14
Plasmids		
pRK600	Cm^r *ori* ColE1 RK2-Mob^+ RK2-Tra^+	7
pGEM7Zf(+)	T7/SP6 cloning and transcription vector	Promega
pMMB67HE	Tac expression cloning vector with cloning sites of pUC18; Ap^r	ATCC
pMMB67HES	pMMB67HE with *PvuII-HindIII* fragment deleted; Ap^r	This study
pMKU101	pUC19with 0.65kb0CSP; Ap^r	7
pMKY341	pT7-5 with *tmoABCEDF*	14, 16
pAM101	pGEM7Xf(+) with *tmoABCEDF*	This study
pAM102	pMKU101 with *tmoABCEDF*	This study
pAM103	pMMB67HES with P*starv1-tmoABCEDF* construct	This study
pAM104	As pAM103 but without P*starv1*	This study

2.2 Construction of plasmids with the *tmo* operon under the control of the P*starv1* promoter

The *tmo* gene cluster was amplified from plasmid pMKY341 (Table 1) by PCR as previously described; Matin et al [13]. The resulting product was flanked by *Apa* I and *Eco* RI restriction sites, enabling it to be cloned downstream of the T7 promoter of pGEM7Zf(+), generating plasmid pAM101 (Table 1). Upon addition of IPTG, the *E. coli* strains (Table 1) bearing this plasmid produced blue-colored colonies on LB agar plates, indicating the successful cloning of the *tmo* operon (the tryptophanase of *E. coli* converts tryptophan present in the LB medium into indole, which in the presence of TMO is converted into deep blue colored indigo).

The *tmo* operon was then excised from pAM101 by *Apa* I and *Eco* RI digestion and cloned into the corresponding sites of pMKU101, which contains the *starv1* promoter; Kim et al [7]. The resulting plasmid, designated pAM102, has the *tmo* gene cluster immediately downstream of the *starv1* promoter. To transfer the P*starv1-tmo* construct to a broad host range plasmid for expression in *P. putida*, we used the plasmid pMMB67HE. Deletion of a ca. 1.5 kb *Pvu* II-*Eco* RI fragment generated the plasmid pMMB67HES, lacking the *tac* promoter and the $lacI^q$ gene. The P*starv1-tmo* construct was excised from pAM102 by *Sph* I and *Eco* RI digestion and transferred to pMMB67HES digested with the same enzymes, generating the plasmid pAM103 (Table 1).

The plasmid pAM103 was transformed into *P. putida* MK1 by triparental mating, as described above, generating strain AMS1001. AMS1001 turned blue upon starvation in glucose-M9 solid or liquid media; since *Pseudomonas* species lack tryptophanase, 1 mM indole was added to these media.

2.3 Northern analysis and transcript mapping

A 283 bp PCR product internal to the *chrR* gene was amplified using the primers CGATGTGGGTTCGCGTCCTTAC and TCAGACCGCCCTGTTCAACTTC, labeled with α-^{32}P ATP (Perkin Elmer) by nick translation, and used to probe total RNA isolated from an early stationary phase culture of *P. putida* KT2440 grown with 0.4 mM chromate.

Transcript mapping was performed using the Promega Primer Extension System (AMV reverse transcriptase) and a Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit from USB. RNA for transcript mapping was isolated from early stationary phase cultures grown with or without 0.4 mM chromate. The primer GATGACCGTTCTCCTGTG, complementary to a region 53 bases upstream of the translational start site of the *chrR* gene, was used to localize the *chrR* promoter.

2.4 Analytical techniques

TMO assay relied on the ability of TMO to convert indole into indigo, which has a deep blue color; Ensley et al [15]. At indicated intervals, 1 ml aliquots of growing cell culture were removed. Indigo was extracted by treatment with an

equal volume of ethyl acetate. Following centrifugation to separate the organic and aqueous phases, the absorbance of the ethyl acetate fraction was measured at 600 nm. The concentration of indigo was determined with reference to a standard curve of indigo dissolved in ethyl acetate; Ensley et al [15].

Crude extracts were prepared as described previously; Ackerley et al [5]. Reaction mixtures contained: 250 μM $FeSO_4$, 2.7 μM FAD, 1 mM NADH, and 400 μM phenol. Appropriate amounts of extract protein were added to the mixture. Reactions were started by the addition of phenol, and incubated at 30 ºC; aliquots were removed at 1 min intervals and analyzed for residual phenol by the antipyrene dye method as described previously; Whited and Gibson [16].

Phenol consumption during growth was also monitored by the antipyrene dye method. TCE degradation and chromate reduction were measured as described by Matin et al [13] and Ackerley et al [5], respectively.

3 Results and discussion

3.1 AMS1001 degrades phenol and TCE in postexponential growth phase with high conversion efficiencies

The presence of 33 mg/L phenol did not affect the growth pattern of the strain AMS1001 (containing the *tmo* operon under control of the P*starv1* starvation promoter): the generation time remained ca. 2 h, and neither the time of entry into different phases nor the final yield were altered. Measurements showed that the exhaustion of glucose from the medium coincided with the end of the exponential phase (data not shown). There was minimal degradation of phenol (ca. 4 mg/L) in the exponential phase; based upon the total growth observed during this transformation, this corresponds to a conversion efficiency of 125 g cell mass generated per mg phenol transformed.

More rapid degradation occurred in the post-exponential phase, resulting in virtually complete conversion of the added phenol (Fig. 1). The highest rate attained was 400 μg phenol degraded/h/g cell dry-weight; ca. 29 mg/L phenol were degraded in this phase. The amount of phenol converted per unit biomass synthesized (the conversion efficiency) was calculated on the assumption that the total growth in the post-exponential phase corresponded to ca. 0.1 mg cell dry wt. Thus, in contrast to the exponential phase, only $3x10^{-5}$ g cell material was generated per mg phenol transformed.

TCE inhibits bacterial growth; McCarty and Samprini [3]; Whited and Gibson [16]. Therefore, in determining TCE degradation by AMS1001, this compound, at a concentration of 1 mg/L, was added at the onset of the post-exponential phase rather than from the start of the culture. The highest rate attained was ca. 110 μg TCE converted/h/g cell dry wt, and a total of 910 μg TCE were transformed. Thus, the conversion efficiency, calculated on the above assumption of biomass synthesis in this phase, was 110 mg cell material generated per mg TCE transformed. With natural populations, the corresponding value is 7 g cell material per mg TCE transformed; Hopkins et al [17]. AMS1004, which bears the plasmid pAM104 (containing the *tmo* operon, but not

P*starv1*), did not exhibit the above conversions, confirming that the expression of TMO in AMS1001 was indeed driven by the starvation promoter, P*starv1*.

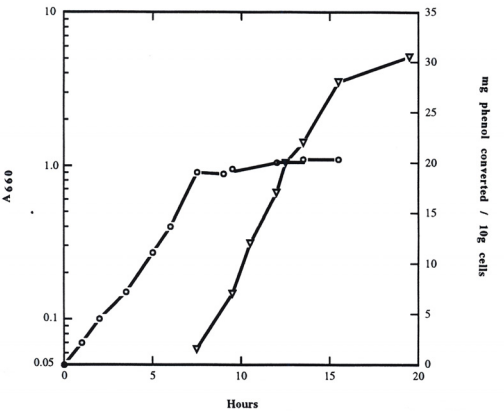

Figure 1: Phenol degradation by AMS1001 in different growth phases in 0.1% glucose-M9 medium. Symbols: (o) A_{660}, (▾) phenol degradation rate.

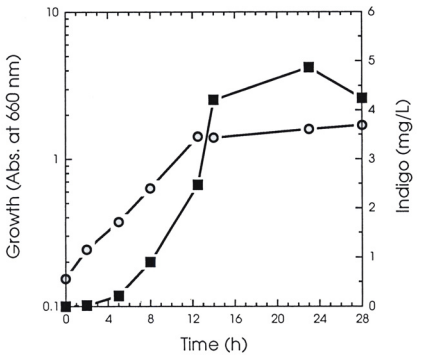

Figure 2: *tmo* expression during exponential and stationary growth phases of *P. mendocina* KR1 in 0.15% glucose M9-medium plus 1 mM indole. Symbols: (o) A_{660}, (■) Indigo.

For comparative purposes, the pattern of TMO expression in *P. mendocina* KR1 in the various growth phases was examined. TMO is apparently an inducible enzyme in this bacterium and phenol can serve as a growth substrate; Yen et al [14]. We therefore examined this pattern in 0.15% glucose-M9 medium supplemented with 1mM indole. The latter can induce TMO but cannot serve as growth substrate. Conversion of indole to indigo in this experiment began with a lag, peaked at the end of the exponential growth and then leveled off (Fig. 2). The results strongly suggest that the native inducible promoter of the *tmo* operon in *P. mendocina* requires rapid exponential growth for expression and is not appreciably expressed in the post-exponential phase.

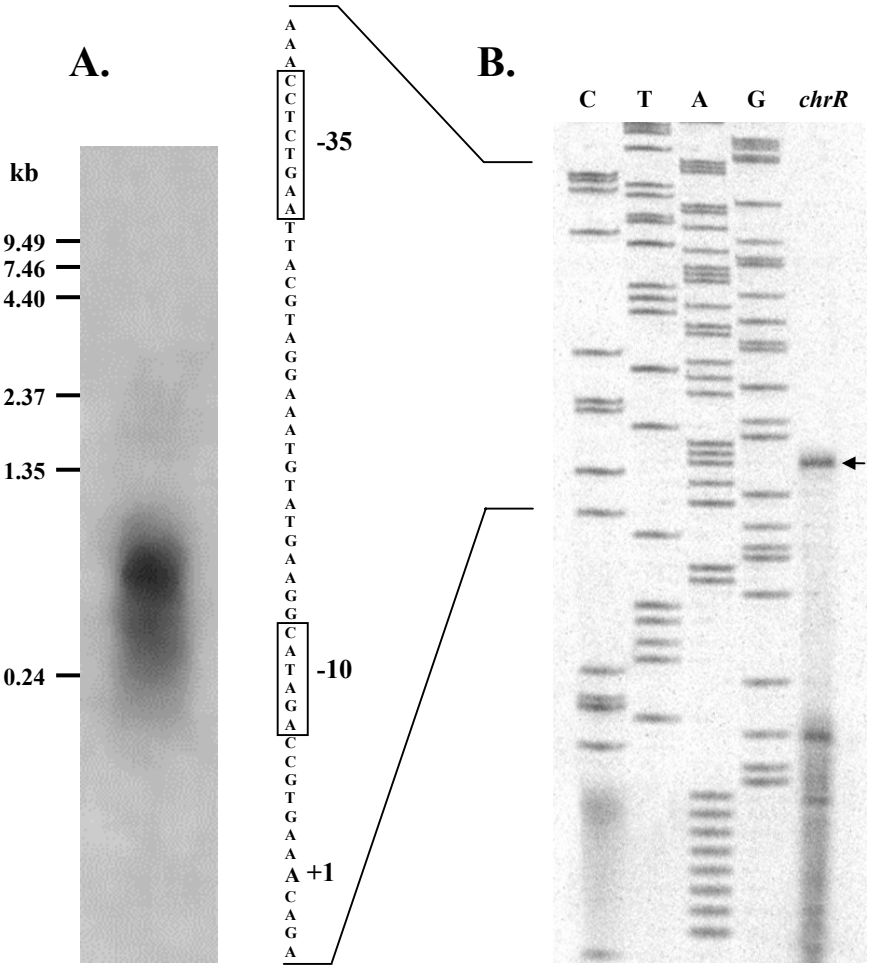

Figure 3: **A.** Detection of the *chrR* transcript by Northern hybridization. Total RNA was isolated from *P. putida* grown to early stationary phase in the presence of 400 μM Cr(VI) and probed with DNA internal to the *chrR* gene. The transcript size was estimated by comparison with ethidium bromide-stained RNA standards. **B.** Mapping of the transcriptional start point of *chrR* by primer extension. Lanes C, T, A, and G show the dideoxy-sequencing ladder obtained with the same oligo used for extension analysis (lane *chrR*). The extension product and corresponding base in the sequencing gel are indicated by an arrow. The nt sequence of the promoter region is shown to the left of the figure, with the proposed -35, -10, and +1 regions highlighted.

3.2 The transcriptional start site and promoter of *chrR*

By Western analysis, we previously showed that ChrR protein attains maximum levels in the stationary phase *P. putida*. This suggests that the native promoter of this gene in this bacterium is a starvation type promoter. If so, the task of improving the native promoter for high level expression during slow growth would be greatly facilitated.

Northern analysis showed that the *chrR* gene transcript was 650 – 750 bases long (Fig. 3A), indicating that this gene is not part of an operon. Twenty three base direct repeats, indicating a strong transcriptional terminator, were identified immediately downstream of the translational stop codon. From these data it was inferred that the transcriptional start site was likely to be located 100-200 bases upstream of the 561 bp *chrR* gene.

The precise transcriptional start site of the *chrR* gene was determined by primer extension analysis (Fig. 3B). The -10 and -35 regions of the *chrR* promoter are similar to the sigma 32 consensus; CATAGA cf CATNTA, and CCTCTGAA cf CCCTTGAA, respectively. It is possible that there is a second transcriptional product, initiated 22 bp downstream of the major transcriptional start site (Fig 3B); however the -10 and -35 regions of this site bear no homology to any known promoters, and the smearing around it suggests that this product is an artifact of primer extension. Transcript mapping of RNA isolated from cells exposed to chromate indicated that there is no alternative promoter activated by chromate challenge (data not shown). Thus, the major promoter regulating *chrR* expression appears to be regulated by sigma 32. The latter is both a heat shock and starvation responsive sigma factor; Jenkins et al [18].

3.3 ChrR activity in low growth rate *P. putida* cell extracts

To obtain more reliable information on *chrR* regulation, we compared chromate reductase activity of rapidly growing cells and those grown at submaximal rates. *P. putida* was grown at different dilution (growth) rates in a chemostat. Steady state cultures were harvested and their total chromate reductase activity measured in cell-free extracts. There was increased activity at low growth rates, the activity being 100 nmoles/min/mg protein at D=0.64 h^{-1}, but increased to 200 nmoles/min/mg protein at D=0.05 h^{-1}. These findings are consistent with *chrR* expression being controlled by a starvation promoter.

Studies currently underway are aimed at further analysis of the promoter region and a search for ancillary factors that may have a role in the regulation of these promoters.

4 Conclusions

1. Genes encoding enzymes that are active in bioremediation may be bioengineered to be under control of starvation promoters; this greatly reduces the amount of biomass formed per unit pollutant transformed. Thus, less nutrients will be required for biostimulation, and clogging will be minimized.

2. The *chrR* gene appears to be expressed from a native starvation promoter, which is highly promising for application of this gene in *in situ* chromate bioremediation.

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