Journal of Industrial Microbiology & Biotechnology (1997) 18, 406–413

Physiological stress in batch cultures of *Pseudomonas putida* 54G during toluene degradation

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Physiological stress associated with toluene exposure in batch cultures of *Pseudomonas putida* 54G was investigated. *P. putida* 54G cells were grown using a continuous vapor phase feed stream containing 150 ppmv or 750 ppmv toluene as the sole carbon and energy source. Cells were enumerated on non-selective (R2A agar plates) and a selective minimal medium incubated in the presence of vapor phase toluene (HCMM2). Differential recovery on the two media was used to evaluate bacterial stress, culturability and loss of toluene-degrading capability. A majority of the bacteria were reversibly stressed and could resume active colony formation on selective medium after passage on non-selective medium. A small fraction of the bacterial cells suffered an irreversible loss of toluene degradation capability and were designated as Tol$^-$ variants. Numbers of stressed organisms increased with duration of toluene exposure and toluene concentration and coincided with accumulation of metabolic intermediates from incomplete toluene degradation. Respiring cell numbers in the batch cultures decreased as injury increased, indicating a possible relationship between respiring and injured cells. Rate expressions for injury, for formation of Tol$^-$ variants and for growth of Tol$^-$ variants were determined by calibrating a theoretical model to the results obtained. These rate expressions can be used to calibrate bioreactor models, and provide a basis for better design and control of bioremediation systems.

Keywords: biodegradation; toluene; *P. putida* 54G; physiological stress; injury

Introduction

The biodegradation of volatile organic compounds (VOCs) such as benzene, toluene and trichloroethylene has been studied extensively for many years [8]. Recovery and enumeration of environmental isolates are often used as measures of biodegradation potential and to evaluate bacterial population dynamics in bioremediation processes for VOCs. In addition, enumeration of isolates derived from contaminated aquifers or soils using minimal media enriched with the VOC of choice, is commonly employed to determine viability of hydrocarbon-degrading bacteria. Visible colonies that form on such a medium are assumed to indicate the population of viable hydrocarbon-degrading cells. Although many studies have shown the presence of environmental bacteria that degrade these compounds, less attention has focused on understanding the physiological effects of isolation and growth on VOCs.

Physiological stress or injury has been defined as the physiological and structural consequence(s) resulting from exposure to sublethal injurious environmental conditions and/or chemical agents [15]. This is reflected by the inability of injured cells to reproduce under selective or restrictive conditions that are tolerated by uninjured cells. The definition and concept of injury include the capability of debilitated cells to repair any cellular damage or other physiological effects and regain tolerance for growth under selective conditions [15]. Consequently, nonselective media must be used to estimate more accurately viable cell numbers exposed to stressors.

The concept of bacterial injury has been studied for over 30 years in the food industry and over 20 years in aquatic environments [16]. An extensive body of experimental evidence is available indicating that a significant proportion of bacteria may exist in a physiologically compromised state variously referred to as ‘stressed’ [1,4] or ‘injured’ [3,5]. Chemicals such as disinfectants and biocides [3,11,14,20,26] and metals such as copper [5,25] have been shown to cause bacterial stress. Physical factors including sunlight [7], UV radiation [18], acidic pH [6,9], heat shock [10,28]and related environmental factors [1] can also significantly contribute to bacterial stress and injury in aqueous systems.

Differential recovery on selective and non-selective growth media has been used to detect and quantify the occurrence of stressed bacteria [1,2,11,19,21]. In studies conducted on indicator bacteria in drinking water distribution systems, the presence of stressed sub-populations has often led to an underestimation of the total viable cells [15]. Injured cells become sensitive to inhibitory agents in specific selective media and are unable to grow and produce colonies [1]. Resuscitation on rich medium before enumeration on selective medium provides a better indication of the total viable cell population.

Only a few studies exist on the topic of hydrocarbon-related stress and injury. Tebbe *et al* [27] used gene probes to differentiate between different categories of operon expression for naphthalene-degrading bacteria and concluded that: (i) some organisms expressed the degradative

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Received 1 July 1996; accepted 25 March 1997

genotype upon selective isolation; (ii) a majority of the organisms only expressed naphthalene degradation after initial non-selective isolation; and (iii) some organisms did not express naphthalene degradation despite confirmation that the genotype was intact. They suggested that the proportion of organisms that immediately expressed their genotype depended on the *in situ* selective pressure imposed on the cells and on their ability to adapt to different nutrient conditions. Love and Grady [13] determined that the continuous culture growth of *P. putida* in the presence of benzoate and *m*-toluate caused cells to lose culturability on benzoate and *m*-toluate plates in comparison to growth in continuous culture using glucose. They suggested that a reversible physiological response could have caused the loss of culturability rather than an irreversible mutation. When cells degrading the hydrocarbon at low concentrations were suddenly exposed to high concentrations, substrate uptake rates increased accordingly. These increased rates led to inhibitory levels of intracellular substrate and/or intermediates that were hypothesized to be responsible for the observed loss in culturability.

Ridgway [23] has studied the growth of hydrocarbon-degrading bacteria from a gasoline-contaminated aquifer and concluded that stressed subpopulations could result in an underestimation of the true number of viable hydrocarbon-degrading bacteria in the aquifer. In a related investigation, Leddy *et al* [12] determined that after 10–15 days of growth on vapor phase toluene, *P. putida* 54G formed Tol^- variants with a selective loss of catabolic functions. The variants could not degrade toluene but continued to grow in the presence of toluene, metabolizing other carbon sources such as organic compounds leaking from wild-type cells or toluene degradation intermediates. They also observed that benzyl alcohol mediated irreversible defects in both a plasmid-associated and chromosome-associated pathway utilized by *P. putida* 54G for toluene degradation.

For successful application of bioremediation processes, it is important to determine how such a stress response can be related to the ability/efficiency of a microorganism to degrade hydrocarbon vapors since these effects might lead to reduced hydrocarbon-degradative activity in bioreactors. The effect of parameters such as hydrocarbon concentration and duration of hydrocarbon exposure on hydrocarbon-associated physiological stress can provide critical information on degradation processes. This information, when obtained in a well-defined system, could be utilized to formulate rate expressions based on growth of stressed and uninjured cells. Establishment of quantitative rate relationships would then permit incorporation of the stress response into process models, leading to an improved ability to design and control bioremediation systems.

We hypothesize that injury in *P. putida* 54G cells is associated with toluene exposure, with a majority of the bacterial cells able to revert to their uninjured state after passage on a rich non-selective medium. A small fraction of the wild-type cells suffer irreversible loss of toluene degradative capability, consequently forming Tol^- variants. Toluene concentration, duration of toluene exposure and formation of intermediates as a result of incomplete toluene degradation, increase bacterial injury. In this investigation, *P. putida* 54G cells were grown in planktonic cultures using toluene as a sole carbon and energy source. Total viable and injured cells were determined for influent vapor phase toluene concentrations of 150 and 750 ppm, respectively. The results of the batch experiments were also used to formulate rate expressions for injury and Tol^- variant formation of toluene-degrading cells and the growth of Tol^- variants.

Material and methods

Bacterial strain

Pseudomonas putida 54G, a toluene-degrading bacterium, was isolated from a gasoline-contaminated aquifer at Seal Beach, California. The isolate was capable of growth on mineral salts medium (containing only inorganic compounds) in the presence of vapor phase toluene and on complex carbon sources such as R2A medium [23]. When grown on mineral salts with toluene, *P. putida* 54G has been shown to exhibit catechol-2,3-dioxygenase activity indicating a *meta* pathway [12].

Media and enumeration of viable cells

HCMM2 mineral salts medium was used as the liquid inorganic nutrient medium as described elsewhere [23]. Plate counts on the following solid media were employed in this investigation:

(1) **R2A** medium [22], which has been used as a non-selective medium for *P. putida* 54G [23];
(2) toluene vapors supplied continuously to cells on HCMM2 plates (selective medium) in sealed containers incubated at room temperature, designated as **HT** plates; and
(3) 300 mg L^{-1} glucose added to HCMM2 plates, designated as **HG** plates. This medium was used to further characterize the nature of any differences observed between HT and R2A culturable cells, if injury consisted solely of loss of the toluene-degrading phenotype, cell numbers on HG should equal those on R2A.

P. putida 54G cultures grown on toluene as the sole source of carbon and energy were enumerated for viable cells on R2A, HT (Table 1) and HG plates. The difference between selective and non-selective media was used to determine culturability according to:

$$\%\ \text{Non-culturable cells} = \left(\frac{\text{Non-selective counts} - \text{Selective counts}}{\text{Non-selective counts}}\right) \times 100 \qquad (1)$$

Colonies that grew on the non-selective medium were transferred aseptically onto the selective medium. Colonies that did not grow on the selective medium after transfer were termed toluene-nondegrading (Tol^-) variants as shown by Leddy *et al* [2]. The number of colonies that grew after transfer were used to calculate the number of injured cells according to:

Table 1 Description of cell enumeration using different microbiological techniques

Description	Enumeration method	Procedure
Total cells	DAPI	Direct microscopic counts of filtered DAPI-stained bacteria
Respiring cells	CTC	Direct microscopic counts of filtered CTC-stained bacteria
Viable cells	R2A	Plate counts on non-selective medium
Toluene-degrading cells	HCMM2 + Toluene (T)	Plate counts on selective medium
Injured cells	R2A → HCMM2 + T	Growth on selective medium after transfer from non-selective medium
Tol^- variants	R2A → HCMM2 + T	No growth on selective medium after transfer from non-selective medium

$$\% \text{ Injured cells} = \left(\frac{\text{Non-selective counts} - \text{Selective counts} - [Tol^-] \text{ variants}}{\text{Non-selective counts}}\right) \times 100 \quad (2)$$

Thus, our definition of injured cells specifically excludes those cells that do not recover the ability to grow on the selective medium.

Microscopy

Total and actively respiring bacteria (Table 1) were determined by direct epifluorescent microscopy. Samples were counterstained by the DNA-binding fluorochrome 2,4-diamidino-2-phyenylindole (DAPI) following reaction with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) based on a procedure outlined by Rodriguez *et al* [24] with minor modifications at final concentrations of 1 mg L^{-1} (DAPI) and 1520 mg L^{-1} (CTC), respectively. Samples withdrawn from the reactor system were diluted in HCMM2 medium, vortexed and homogenized (Model TR-10, 60% of maximum power, Tekmar Co, Cincinnati, OH, USA). The samples were then stained with CTC and incubated at room temperature with stirring for 2 h, then filtered on 0.2-μm pore-size black polycarbonate membrane filters (Poretics Corp, Livermore, CA, USA). The samples were washed with autoclaved distilled water and stained with DAPI on the filter for 40 min, followed by two more washing steps. Filters were air-dried and placed on glass slides for microscopic examination using an Olympus BH2-RFCA epifluorescent light microscope (Scientific Instrument Company, Aurora, CO, USA) with a 100× (refractive index = 1.3) oil immersion lens. Cells that stained with CTC appeared red (indicating actively respiring cells) while inactive cells appeared green and provided a measure of total (respiring + inactive) cells. The fraction of respiring cells was then calculated as the ratio of the CTC to DAPI counts.

A summary of the nomenclature for the various populations and the method for their determination is presented in Table 1. Due to inherent differences between plating techniques and microscopic count methods, no attempt was made to correlate counts from the two different techniques.

Toluene sampling

Gas and liquid samples were analyzed for toluene using gas chromatography (GC). In the headspace, toluene was measured using a HP 5890 Series II GC equipped with a flame-ionization detector and an Alltech 0.1% A1-1000 Graphpac GC 80/100, 1.8 m × 3.2 mm × 2.2 mm, stainless steel column at a constant column temperature of 140°C. Headspace samples (250 μl) were withdrawn and injected into the GC. Toluene concentration in the liquid was determined by combining 1 ml of the aqueous samples with 0.5 ml of hexane and injecting 2 μl of the hexane phase into a HP 5890 Series II GC equipped with a photo-ionization detector and a DB-624 column (30 m long × 0.53 mm inside diameter) which was temperature ramped from 140–200°C. Helium was used as a carrier gas in both columns at a flow rate of 30 ml min^{-1}.

Measurement of non-volatile soluble intermediate

Liquid samples were withdrawn from the system and combined with methylene chloride to extract toluene. Aliquots (2 μl) of the methylene chloride phase were injected into a HP 5890 Series II Plus GC combined in series with a VG Analytical GC-MS 70E-HF Double Focusing Mass Spectrometer (MS). A HP-5 capillary column with dimensions of 30 m × 0.25 mm i.d. and a film thickness of 0.25 μm was used in the GC. The entire sample was transported to the MS using helium as a carrier gas at a flow velocity of 30 cm s^{-1}.

Experimental system and design

A manifold system (Figure 1) was used to measure bacterial injury and non-culturability associated with exposure to

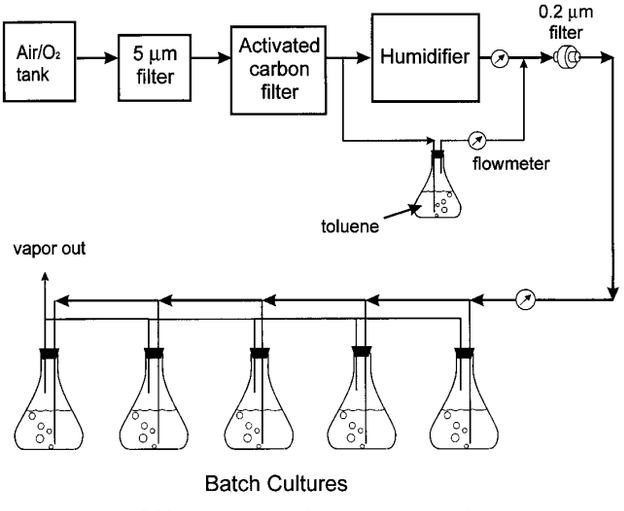

Figure 1 Manifold system to produce continuous toluene exposure at constant concentrations.

constant concentration of toluene. The reactor system allowed for *in situ* measurement of toluene in the gas and liquid phases. Liquid batch cultures of *P. putida* 54G were grown with toluene as sole carbon and energy source under three different constant experimental conditions.

Experiment 1: An arbitrary vapor phase toluene concentration of 300 ppm was used with viable cell numbers enumerated on HG, HT and R2A plates. Colony transfers and microscopic techniques were not used in this experiment. R2A plates have been used as a non-selective medium but we wanted to determine whether resuscitation could occur solely by replacing the toluene with glucose, eliminating the stress imposed by toluene. Similar counts on R2A and HG plates would be indicative of a stress mechanism specific to the toluene pathway; conversely, if cell numbers on HG and HT plates were similar, then a less specific mechanism would be involved. In the latter case, R2A plates alone would be used as a non-selective medium for all subsequent experiments.

Experiment 2: Vapor phase toluene was fed at a concentration of 150 ppm with viable cell numbers enumerated on HT and R2A plates, and total and actively respiring cells enumerated by staining with DAPI and CTC as explained above.

Experiment 3: Vapor phase toluene was fed at a concentration of 750 ppm with cells enumerated as in Experiment 2.

Air flow was separated into two streams with one stream flowing into a humidifier and the other flowing into an Erlenmeyer flask containing liquid toluene. For Experiment 3, oxygen was used as a carrier gas for toluene to ensure that oxygen was not stoichiometrically limiting. Flasks were operated abiotically for 5 days and oxygen and toluene concentrations were measured. The two streams were mixed and flowed into 400-ml flasks where they bubbled through 250 ml of HCMM2 medium. Gas effluent from the flasks flowed into a chemical hood and was vented into the atmosphere. Flasks were equipped with sampling ports containing Teflon-lined septa and were stirred continuously to ensure sufficient aeration. At the start of the experiment, cells of *P. putida* 54G, pregrown on toluene vapors, were inoculated into the flasks. At timed intervals, toluene concentrations in the gas and liquid phases, cell numbers and non-volatile intermediates were measured by sampling the flasks.

Results

Test for HG as a non-selective medium

An arbitrary influent toluene vapor phase concentration of 300 ppm was chosen for this study. At timed intervals, liquid samples were withdrawn from the flasks and enumerated on R2A, HT and HG plates (Figure 2). Cell numbers on HG and HT plates show a similar pattern, wherein there was an initial increase in viable cell numbers during log growth phase until the end of day 1. During this period, cell numbers increased by four orders of magnitude from about 10^5 to nearly 10^9 colony forming units per milliliter

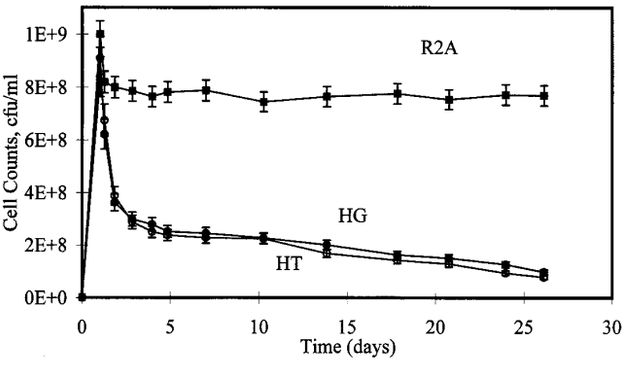

Figure 2 Cell numbers on R2A plates, HCMM2 plates combined with glucose (HG) and HCMM2 plates incubated in the presence of vapor phase toluene (HT).

(CFU ml^{-1}) for both HT and HG plates. This was followed by a decrease in cell numbers to fewer than 10^8 CFU ml^{-1}, which continued until the end of the experiment (27 days). No significant difference was measured between cell numbers based on growth on HT and HG plates. Cells on R2A plates increased to 1×10^9 at day 1, then decreased slightly to 8×10^8 CFU ml^{-1} and stayed at that level for the duration of the experiment. Based on cell numbers on R2A and HT plates, % non-culturable cells was calculated as 89% (from Equation 1). The loss of culturability on both HT and HG plates compared to R2A led us to conclude that HG could not be used as a non-selective medium. We also tested HCMM2 + glutamate as a potential non-selective medium and obtained essentially the same result (data not shown). From these results, we concluded that the presence of toluene on the selective medium was not the sole factor leading to decreased recovery; at least one of the wide variety of organic nutrient sources available on R2A was required for rescuscitation. At this point, we decided to use R2A plates as a non-selective medium and HT plates as a selective medium for all subsequent injury experiments.

Injury with 150 ppm feed

Liquid samples were analyzed for viable cells on HT and R2A plates. Total cell numbers and actively respiring cells were estimated using microscopic techniques combined with DAPI and CTC staining, respectively. The progression of cell numbers (viable and total) in the manifold system is shown in Figure 3. Each data point represents an average of three values with each experiment performed in duplicate. The maximum standard error for these values was approximately 12%, but averaged 5%. There was an initial rise in cell numbers during the log phase followed by a stationary phase characterized by a relatively constant number of total cells (DAPI) and 'viable' cells (R2A). Cell numbers on HT plates showed a steady decline from $(4 \pm 0.43) \times 10^8$ CFU ml^{-1} at 0.833 days to $(5.1 \pm 0.17) \times 10^7$ CFU ml^{-1} at 29 days. Although numbers of CTC-stained cells and viable cells on R2A plates were similar, CTC counts decreased slowly over the period from day 2 to day 29. During this study, we also sampled the flasks to determine pH and inorganic nutrient (dissolved nitrate, sulfate and phosphate) concentrations. The inorganic nutrient concentrations decreased to approximately

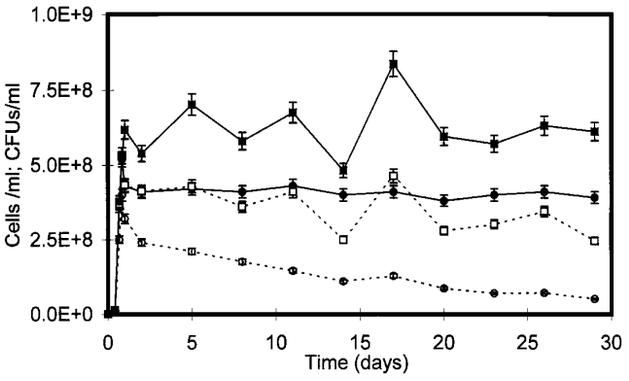

Figure 3 Progression of cell numbers during the study which used 150 ppm toluene determined by staining with CTC (--□--) and DAPI (-■-) in cells ml^{-1}, and viable cell numbers in CFU ml^{-1} obtained on R2A plates (-●-) and HCMM2 plates incubated in the presence of vapor phase toluene (HT) (--○--).

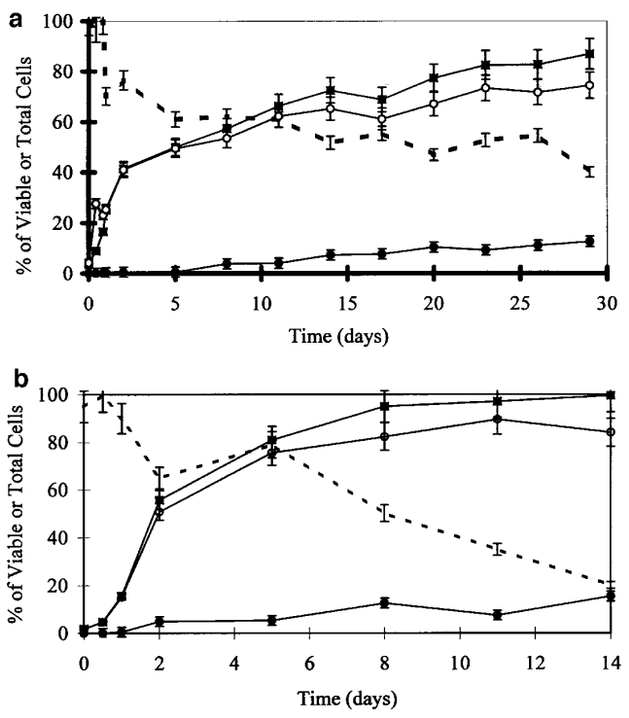

Figure 4 Percentage non-culturable cells (■), injured cells (○) and variants (●) compared to viable cells, and respiring cells (- -) compared to total cells during the (a) 150 ppmv study and (b) 750 ppmv study.

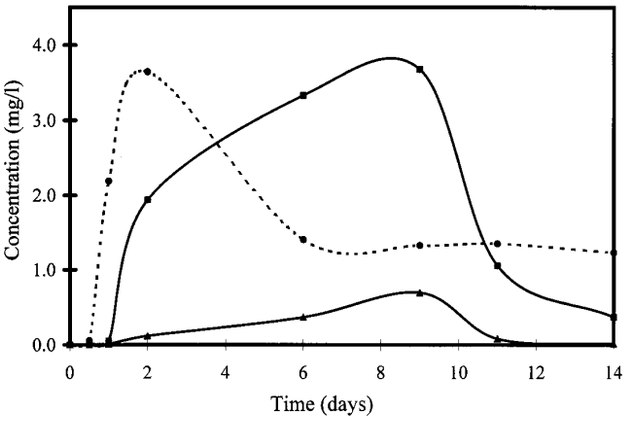

Figure 5 Accumulation of intermediates due to incomplete toluene degradation during the study which used 750 ppm toluene. --●--, Benzyl alcohol; -■-, benzoate; -▲-, benzaldehyde.

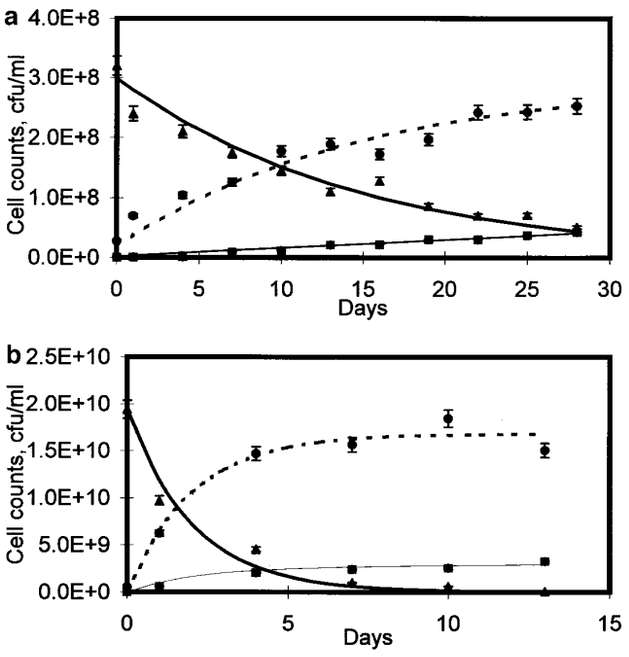

Figure 6 Injury model fitted to results obtained from the study which used (a) 150 ppm toluene and (b) 750 ppm toluene. Markers indicate experimental results and lines represent model predictions for toluene-culturable cells (▲ and - -), injured cells (● and heavy solid line) and variants (■ and light solid line).

50% of their initial level after 29 days of toluene exposure and the pH decreased from 7.2 to approximately 6.3 over the same period.

The difference in cell numbers on R2A and HT plates was used as a measure of non-culturability that increased from 3.5% at the start of the experiment to 87% after 29 days (Figure 4a). Based on this study it was apparent that the degree of non-culturability was related to duration of toluene exposure. Colonies that formed on non-selective R2A medium were transferred aseptically onto selective medium (HT plates). Colonies that did not grow on the selective medium were termed Tol^- variants and the fraction that grew on the selective medium were termed injured. Figure 4a shows that the fraction of Tol^- variants increased to 12.6% after 29 days of toluene exposure, while the injury fraction accounted for 74.4% of the cells.

Respiration % was calculated as the ratio of CTC- to DAPI-stained cells and indicated the fraction of cells that were respiring (Figure 4a). Respiring cells decreased from 100% at the start of the experiment to about 35% at the end of the experiment, at day 29.

Injury with 750 ppm feed

The manifold system was operated at an influent vapor phase toluene concentration of 750 ppm with the same enumeration procedures as in Experiment 2. During the 14-day

experiment, cell enumerations provided qualitatively similar results to those shown in Figure 3, but counts that were 1–2 orders of magnitude higher. The greatest differences were in cell numbers on HT plates, which increased to $(1.94 \pm 0.22) \times 10^{10}$ CFU ml^{-1} during log phase and then decreased continuously to $(7.8 \pm 0.59) \times 10^{7}$ at day 14. Correspondingly, the non-culturable cell fraction increased from 1.8% at the start of the experiment to 99.6% after day 14 (Figure 4b). Tol$^-$ variants increased from 0 to 15.5% by day 14, while the injured fraction concurrently increased from 1.8% to 84.1%. The respiring fraction of total cells decreased from 95% to about 19% at the end of the 14-day experiment.

Liquid samples were withdrawn for identification of soluble non-volatile metabolites. Three principal compounds were identified: benzyl alcohol, benzaldehyde and benzoate (Figure 5). Benzyl alcohol increased to 3.5 mg L^{-1} at day 2 and then decreased to a constant value of approximately 1.5 mg L^{-1}. Benzaldehyde and benzoate increased more gradually and then decreased more rapidly to less than 0.5 mg L^{-1}. All three compounds are associated with a plasmid-associated toluene degradation pathway for *P. putida* 54G [12]. We also noticed accumulation of *o*-cresol (data not shown) during the experiment which could not be associated with known toluene degradation pathways of *P. putida* 54G. None of these intermediates were detected in abiotic controls run over 5 days.

Rate expressions for toluene-degrading cells, injured cells and Tol$^-$ variants

Rate expressions for toluene-degrading cells (X_{td}), injured cells (X_i) and Tol$^-$ variants (X_n) were determined by formulating mass balances for each cell type in a batch reactor system starting at the end of the log growth phase. For example, a mass balance on X_{td} cells included growth, injury, irreversible loss of toluene degradative capability and decay. Similar mass balances were written for X_i and X_n cells and the resulting set of coupled differential equations were simplified using the following assumptions:

(1) Net growth of injured cells was negligible because they had to be resuscitated on rich mineral medium prior to degrading toluene.
(2) The net growth of toluene-degrading cells was negligible in comparison to other processes that toluene-degrading cells undergo, namely irreversible loss of toluene-degrading capability and injury.
(3) The difference between the growth and decay rates of Tol$^-$ variant cells could be combined into one variable, μ_{NET}, which expressed the net growth rate of X_n cells. This process was included based on Leddy *et al* [12], who demonstrated growth of confirmed Tol$^-$ variants in media fed only toluene.

Based on these assumptions, mass balances for the different cell types were simplified and solved analytically to yield the following solutions (Equations 3, 4 and 5):

$$X_{td} = X_0 \exp[-(K_I + K_{GL})t] \quad (3)$$

$$X_i = \frac{K_I}{K_I + K_{GL}} X_0 (1 - \exp[-(K_I + K_{GL})t]) \quad (4)$$

$$X_n = \frac{K_{GL} X_0 \exp[\mu_{NET} t]}{(K_I + K_{GL} + \mu_{NET})} (1 - \exp[-(K_I + K_{GL} + \mu_{NET})t]) \quad (5)$$

In Equations 3, 4 and 5, K_I is an injury coefficient [day^{-1}], K_{GL} is an irreversible loss coefficient [day^{-1}] and X_0 is the maximum number of uninjured cells formed at the end of the logarithmic growth phase (time 0). These equations were solved using the initial condition that the initial number of X_i and X_n cells were zero, and X_{td} cell numbers were X_0. The Equations were fitted (Figures 6a and b) to results from the 150- and 750-ppm studies to obtain numerical values for the three parameters, K_I, K_{GL} and μ_{NET} (Table 2). As seen in Table 2, K_I and K_{GL} increased as influent vapor phase toluene concentration was increased, while μ_{NET} decreased by a factor of 0.5. The ratios of K_I and K_{GL} for Experiment 3 (750 ppmv) to their values in Experiment 2 (150 ppmv) were approximately 6 and 10, respectively. With a five-fold increase in toluene concentration, the rate of injury increased by a factor of six and irreversible loss of toluene-degradative capability increased by a factor of ten.

Discussion

The results of this investigation demonstrate that hydrocarbon-degrading bacteria may undergo a physiological stress response that can be quantified via differential plating on selective minimal and non-selective media. Growth on a selective medium does not necessarily represent a true estimate of the viable cell numbers and this discrepancy can be explained on the basis of a stress response of the bacteria to sublethal physiological and selective pressure. Two types of cells, injured and Tol$^-$ variants, were formed during continuous exposure of *P. putida* 54G cells to toluene vapors. The injured cells suffered a repairable stress-related defect which was reversed by passage on R2A medium. The Tol$^-$ variants suffered irreversible loss of toluene-degrading capability, and could not grow on selective medium even after resuscitation on R2A.

The choice of non-selective medium is critical when determining bacterial injury. In this study, both glucose and glutamate proved unsuitable as the sole organic nutrients for non-selective media since *P. putida* 54G showed lower recovery on both media in comparison to recovery on R2A plates. This result is in contrast with

Table 2 Parameters obtained by fitting theoretical models to results from the studies involving feed rates of 150 and 750 ppm toluene

Parameter	Vapor feed concentration	
	150 ppm	750 ppm
X_0, CFU ml^{-1}	3.2×10^8	1.94×10^{10}
K_I, day^{-1}	0.0678	0.4269
K_{GL}, day^{-1}	0.0065	0.0655
μ_{NET}, day^{-1}	0.0255	0.0118

those of Love and Grady [13], who determined that rich media (10 g L^{-1} tryptone, 1 g L^{-1} yeast extract) produced about the same recovery of injured cells as medium containing only 700 mg L^{-1} glucose and mineral nutrients. The loss of culturability in our study was due to more than the intracellular accumulation of substrate and metabolites observed by Love and Grady [13]; simply replacing the toluene with a 'non-hazardous' substrate (glucose) was insufficient for recovery of culturability. In this context, appropriate non-selective media should be evaluated for each bacterium before experiments to assess bacterial injury are carried out. Similarly, counts on selective medium may significantly underestimate the number of organisms that are capable of metabolizing a target compound; colony transfers from non-selective to selective medium are required to evaluate this potential.

Cells that exhibited irreversible loss of toluene-degradative capacity increased continuously throughout the duration of the experiment. While we did not determine if the Tol^- variants were growing, Leddy *et al* [1] hypothesized that the variants possibly grew on trace organics leaking from wild-type cells grown on toluene. The accumulation of intermediates such as benzyl alcohol has been shown to promote formation of Tol^- variants [12]. In the present investigation, three principal intermediates were formed: benzyl alcohol, benzaldehyde and benzoate. The increase of benzyl alcohol at day 2 coincided with an increase in injury (Figure 4), and approximately 1.5 mg L^{-1} benzyl alcohol was present throughout the remainder of the study. It is quite possible that the injured cells degrade toluene incompletely leading to an increase in the formation of the intermediates.

As the level of injury observed in batch cultures increased, the fraction of respiring cells decreased (Figure 4). While these results do not conclusively prove a relationship between injury and respiration, in the absence of any dramatic changes in pH or other growth conditions it is possible that the increase in the number of injured cells may be related to the decrease in numbers of respiring cells. We have measured stratified layers of respiratory activity in *P. putida* 54G biofilms during toluene degradation [17]. These stratified layers could be regions of injured cells, toluene-degrading cells and variants which stratify depending on the presence of substrates that they can metabolize. For an improved understanding of bacterial activity in bioreactors, physiological effects of injury on respiration rates need to be evaluated.

Rate expressions evaluated from calibrating a theoretical injury model to experimental results yielded values of injury and irreversible loss coefficients. While these models fall short of describing actual physiological changes in the cells, several observations from this effort remain significant. As toluene concentration was increased five times, the injury coefficient, K_I, increased by a factor of six and the irreversible loss coefficient, K_{GL}, increased by a factor of ten. These rate expressions suggest that increasing toluene concentration resulted in a substantial increase in bacterial injury in response to inhibitory conditions in the batch cultures. As toluene exposure is prolonged, injured and Tol^- variant cells continue to accumulate, eventually negating the growth of competent cells.

There are a number of justifications for the enumeration of injured bacteria in environmental systems related to groundwater pollution and remediation. Most isolation techniques involve selective media which frequently underestimate numbers of viable hydrocarbon-degrading bacteria due to the microorganisms' long-term exposure to sub-optimal growth conditions. It is also imperative to determine if injury in biofilm systems is different from injury caused to planktonic bacteria. The ramifications of such a stress response are numerous and need to be recognized to improve the design of remediation systems and assessment of bioremediation potential.

Acknowledgements

This work was supported by the Center for Biofilm Engineering, a National Science Foundation-sponsored Engineering Research Center (cooperative agreement EEC-8907039), Orange County Water District and National Water Research Institute.

We thank Eva Kreiger and Menu Leddy for invaluable guidance and suggestions, Venkat Reddy, Fernando HeDaro and Lance Chatwell for assisting during the experiments, and Joe Sears for the expert technical support with the GC-MS.

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