Activity and Stability of a Recombinant Plasmid-Borne TCE Degradative Pathway in Suspended Cultures

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Abstract: The retention and expression of the plasmid-borne, TCE degradative toluene-*ortho*-monooxygenase (TOM) pathway in suspended continuous cultures of transconjugant *Burkholderia cepacia* 17616 (TOM_{31c}) were studied. Acetate growth and TCE degradation kinetics for the transconjugant host are described and utilized in a plasmid loss model. Plasmid maintenance did not have a significant effect on the growth rate of the transconjugant. Both plasmid-bearing and plasmid-free strains followed Andrews inhibition growth kinetics when grown on acetate and had maximum growth rates of 0.22 h^{-1}. The transconjugant was capable of degrading TCE at a maximum rate of 9.7 nmol TCE/min · mg protein, which is comparable to the rates found for the original plasmid host, *Burkholderia cepacia* $PR1_{31}$ (TOM_{31c}). The specific activity of the TOM pathway was found to be a linear function of growth rate. Plasmid maintenance was studied at three different growth rates: 0.17/h, 0.1/h, and 0.065/h. Plasmid maintenance was found to be a function of growth rate, with the probability of loss ranging from 0.027 at a growth rate of 0.065/h to 0.034 at a growth rate 0.17/h. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **57:** 287–296, 1998.

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INTRODUCTION

Remediation of soil and ground water contaminated by organic pollutants has been the focus of much research in recent years. Some of the most ubiquitous contaminants in America's ground water aquifers are volatile organics, a large number of which are chlorinated aliphatic compounds, including the EPA priority pollutant, trichloroethylene (TCE). Many biological methods have been developed to detoxify TCE-laden soil and ground water, including anaerobic dechlorination (Bouwer and McCarty, 1983; Ewers et al., 1990; Fogel et al., 1986; Freedman and Gossett, 1989; Kleopfer et al., 1985; Vogel and McCarty, 1985) and oxygenase-driven TCE degradation (Alvarez-Cohen and McCarty, 1991a,b; Arciero et al., 1989; Finette et al., 1984; Kaphammer et al., 1990; Nelson et al., 1986; Oldenhuis et al., 1989; Shields and Reagin, 1992; Tsien et al., 1989; Vandenbergh and Kunka, 1988; Wackett and Gibson, 1988; Wackett et al., 1989; Winter et al., 1989). One of the most notable aerobic, cometabolic TCE degrading bacterial species is the environmental isolate *Burkholderia cepacia* G4 (formerly *Pseudomonas cepacia* G4) (Nelson et al., 1986). *B. cepacia* G4 degrades TCE via a cometabolic pathway that must be induced by a cosubstrate, either phenol, toluene, *o*-cresol, *m*-cresol, or catechol (Nelson et al., 1987, 1988).

In laboratory studies performed by Shields et al. (1992), Tn5 transposon mutants of *B. cepacia* G4 were developed that are capable of constitutive mineralization of TCE. The genesis of these transposon mutants is depicted in Figure 1. The transposon mutation resulted in two phenol revertants (*B. cepacia* $PR1_{31}$ [TOM_{31c}] and *B. cepacia* $PR1_{23}$ [TOM_{23c}]), both which were capable of constitutive degradation of TCE. One of these transposon mutants, *B. cepacia* $PR1_{31}$ (TOM_{31c}), carries the complete Tn5 transposon, including kanamycin (Km) resistance, on the toluene/TCE degradative plasmid TOM_{31c}.

The utility of TOM_{31c} for TCE degradation in either open ecosystems or closed bioreactor bioremediation systems rests on the ability to find an appropriate host for the plasmid. A suitable host for TOM_{31c} must have a resistance to waste-related cell injury and toxicity, an ability to retain and express the desired plasmid-borne phenotype during long-term operation, and an ability to persist in a desired ecosystem or bioreactor environment.

Microorganisms carrying plasmids are susceptible to plasmid instability which can lead to the loss of a desired plasmid-borne phenotype or complete loss or inactivation of a desired plasmid-borne genotype. Plasmid instability can occur in two ways: (1) segregational instability; and (2)

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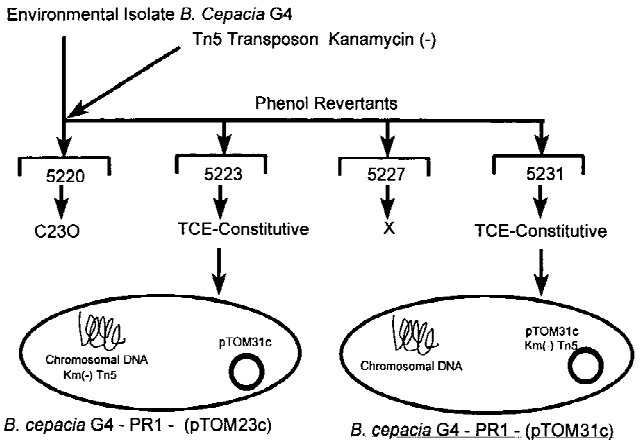

Figure 1. Genesis of plasmid TOM_{31c} via the Tn5 transposon mutagenesis of *Burkholderia cepacia* G4 to produce *Burkholderia cepacia* $PR1_{31}$ (TOM_{31c}) (Shields and Reagin, 1992).

structural instability. Segregational instability is the consequence of random and irregular partitioning of plasmids to both daughter cells during cell division, and can lead to new generations of daughter cells that do not contain the plasmid. Structural instability of a plasmid involves the actual change or recombination (deletion, insertion, and/or rearrangement) of a single gene or several genes on the plasmid. Structural instability can also occur when a portion of the plasmid DNA is incorporated into the chromosomal DNA. In addition, structural instability may involve the loss of a certain plasmid-borne phenotype, but not the loss of other phenotypes associated with the plasmid. For instance, structural instability of plasmid TOM_{31c} could result in the loss of the TCE degrading phenotype, but not the loss of the plasmid-borne, Tn5-encoded, kanamycin resistance.

Much published research on the stability of plasmids exists. Table I lists some of the factors affecting plasmid retention and stability. A great deal of this research has focused on suspended cell cultures and the effects of continuous culture parameters on plasmid stability (Dwilvedi et al., 1982; Dykhuizen and Hartl, 1983; Ensley, 1986; Jones et al., 1980; Kadam et al., 1987; Kumar et al., 1990, 1991; Noack et al., 1982; Ollis, 1982; Roth et al., 1980; Roth and Noack, 1982; Wood et al., 1990). It is important to understand plasmid/host interactions to utilize recombinant plasmids in environmental remediation systems. In addition, the use of plasmid recombinant microorganisms, such as *B. cepacia* 17616 (TOM_{31c}), in open ecosystems mandates research into the fundamental plasmid/host processes that influence plasmid retention, stability, expression, and transfer.

Transconjugant *B. cepacia* 17616 (TOM_{31c}) was used in all experiments to evaluate plasmid maintenance, expression, and stability in suspended cultures. Plasmid TOM_{31c} was chosen for these studies for three reasons: (1) TOM_{31c} is capable of constitutively degrading TCE and thus has obvious applications to the bioremediation of TCE-laden air, soil, and ground water; (2) TOM_{31c} originates from an environmental isolate and is not considered an industrial or genetically designed plasmid; and (3) TOM_{31c} is easily selected for, using either kanamycin (resistance on plasmid-borne Tn5 insertion, see Fig. 1) or various specific hydrocarbons as growth substrates. 17616 was chosen to host TOM_{31c} because it has been well characterized (Cheng and Lessie, 1994) and it is relatively easy to work with and maintain.

This article reports on the laboratory evaluation of the retention and expression of the TCE degrading plasmid TOM_{31c} in the transconjugant host *B. cepacia* 17616 in suspended cultures. A subsequent article examines the retention and expression of the recombinant TCE degrading plasmid in *B. cepacia* biofilm cultures (Sharp et al., 1997).

MATERIALS AND METHODS

Plasmid/Host System

Plasmid-bearing strains of *B. cepacia* were obtained by solid surface conjugation between $PR1_{31}$ (TOM_{31c}) and 17616. Cultures of the original plasmid host, $PR1_{31}$ (TOM_{31c}), were used in a number of studies as either controls or for comparison with 17616 (TOM_{31c}) cultures. Glycerol/peptone frozen cultures (−70°C) of plasmid-free and plasmid-bearing cultures were maintained and used for

Table I. Factors affecting plasmid stability and retention.

Environmental and physiological factors	References
Growth rate. Increases plasmid loss with increased growth rate.	Stewart and Carlson (1986); de Taxis du Poet et al. (1987); Seo and Bailey (1985)
Plasmid copynumber. Decreased plasmid loss rate with increased plasmid copynumber. Plasmid copynumbers can range from 1 to 700.	Huang et al. (1993); Sayadi et al. (1989); Uhlin and Nordstrom (1978); Peretti and Bailey (1987)
Carbon-to-nitrogen ratios. Increased nitrogen growth conditions can increase plasmid stability.	Huang et al. (1994); Sayadi et al. (1989)
Selection. Selection of plasmid using a selective carbon source or antibiotic resistance markers can increase plasmid retention in a given population.	Lauffenburger (1987); Tiedje et al. (1989); Wood et al. (1990)
Nutrient limitations. Nitrogen, phosphorus, potassium, magnesium, and carbon limitations may either increase or decrease plasmid stability.	Godwin and Slater (1979); Jones and Melling (1984); Noack et al. (1982)
Immobilization and attachment. Plasmid-bearing populations that are immobilized or in a biofilm culture may display either increased or decreased plasmid stability.	Huang et al. (1993); Kumar and Schugerl (1990); Inloes et al. (1983); Dykhuizen and Hartl (1983)
Exposure to injurious or toxic substances. Injury and toxicity may lead to increased plasmid loss.	Ridgway (personal communication, 1994); Sharp (1995)

starter cultures in all of the experiments. Selective phenol-kanamycin agar plates of all plasmid-bearing cultures were maintained and restreaked every week, as were phenol-kanamycin slants, which were restreaked from frozen culture every month. All batch and continuous culture experiments were inoculated with a culture of 17616 (TOM_{31c}) cells harvested from highly selective starter cultures (phenol HCMM2 medium with 80 μg/mL of Km). In addition, all continuous flow experiments began with 80 μg/mL of kanamycin in the initial reactor volume to insure each experiment started with a pure plasmid-bearing culture.

Media

Three different media were used in this research: (1) rich general growth medium—Luria broth glucose (LBG; 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 1 g/L glucose); (2) nonselective medium, hydrocarbon minimal medium (HCMM2) amended with the nonselective carbon source, acetate (0.5 to 40 m*M* sodium acetate); and (3) selective medium, HCMM2 amended with phenol (2 m*M*) and kanamycin (50 μg/mL). Acetate was chosen as the nonselective growth substrate because both 17616 (TOM_{31c}) and $PR1_{31}$ (TOM_{31c}) grow well on acetate, it is easily analyzed using ion chromatography, and it does not competitively inhibit TCE co-oxidation. All nonselective agar plates were made with 15 g/L bacto-agar (Difco), whereas all plasmid-selective plates using phenol were made with low carbon Noble agar (Difco) to ensure that phenol was the sole growth substrate.

TOM Specific Activity Analyses

The *m*-trifluoromethylphenol (TFMP) assay was used to indicate the expression and activity of both toluene *ortho*-monooxygenase (TOM) and catechol 2,3-dioxygenase (C230) (Shields et al., 1989), which make up the TOM pathway. The TFMP assay is a colorimetric assay, which involves the transformation of TFMP to TFM-catechol (TFMC) via TOM, followed by the conversion of TFMC to 7,7,7-trifluoro-2-hydroxy-6-oxo-2,4-heptadienoic acid (TFHA) via C230. TFHA is a bright yellow product (adsorption maximum = 386 nm) that is quantified using color spectrometry (A386) with a relatively high extinction coefficient (E_{386} = 26,900) (Engesser et al., 1988).

The TFMP assay is used to determine the presence of TOM_{31c} through the activities of two TOM-encoded enzymes. The TFMP assay was performed using two methods: (1) a colony assay used to make a positive/negative determination of TOM expression in single colonies (Shields et al., 1991); and (2) a suspended culture assay used to quantify the specific activity of the TOM pathway in suspended cultures (Sharp, 1995).

Suspended culture TFMP assay results represent the activity, or rate of TFHA production, of the TOM pathway borne on the plasmid TOM_{31c} per unit of bacterial culture. These results are reported as either specific TOM activity of the whole culture, referred to as ''total TFMP activity'' (milligrams TFHA produced/milligram total biomass per minute), or as specific TOM activity relative to just the plasmid-bearing fraction of a culture, referred to as ''true TFMP activity'' (milligrams TFHA produced/milligram plasmid-bearing biomass per minute).

TCE Degradation Rate Studies

TCE Disappearance Studies

Plasmid TOM_{31c} activity and expression was determined using batch reactor TCE disappearance assays. Assays were carried out as per Folsom et al. (1990), using 25 mL of 17616 (TOM_{31c}) culture harvested from a 2 m*M* phenol-HCMM2 continuous culture. TCE degradation rate constants for relevant TCE concentrations were determined for 17616 (TOM_{31c}) by running a series of TCE disappearance assays at different TCE concentrations (5 to 60 μ*M* in solution). The initial specific TCE degradation rates (nanomoles TCE utilized per minute per milligram protein) were determined at several discrete TCE concentrations and plotted as specific TCE degradation rate versus TCE concentration. The data were assumed to follow Michaelis–Menten kinetics. A Henry's law constant of 0.38 (± 0.03) [mol TCE vapor phase]/[mol TCE solution phase] was determined and used for the 17616 (TOM_{31c}) batch cultures. Appropriate cell-free and inactivated cell controls, and TCE calibration curves were used for each experiment and in determining the dimensionless Henry's law constant.

TCE Analysis

TCE analysis was performed using a Shimadzu Gas Chromatograph (Model GC-9a) equipped with a Shimadzu electron capture detector (ECD) and a 30-m, 0.53-mm-i.d. Vocol megabore column (Supelco, Inc). The analytical method was isothermal (110°C oven temperature, 200°C detector and injector temperature) with a carrier gas flow of 5 mL/min and a make-up gas flow of 50 mL/min. Ultrahigh-purity nitrogen was used as both the carrier and make-up gas. Vapor phase TCE samples were injected directly onto the column and liquid samples were extracted with pentane and then analyzed.

Acetate Growth Kinetics

The metabolic demand of maintaining the plasmid TOM_{31c} was determined by comparing acetate growth kinetics for both plasmid-free and plasmid-bearing 17616 cultures. Acetate growth kinetics (biomass and acetate concentrations over time) were determined from batch reactor data analyzed using an initial rate method (D'Adamo et al., 1984), where the initial acetate concentrations ranged from 0.5 to 50 m*M* diluted in HCMM2 medium. Each acetate batch reactor study was run in triplicate with appropriate cell-free

and inactive-cell controls. Resultant rates versus initial acetate concentrations were correlated using Andrews inhibition kinetics (Andrews et al., 1968). Kinetic constants from this correlation were used both to evaluate plasmid metabolic demand on the growth rate of transconjugant 17616 (TOM_{31c}) and in the implementation of a plasmid loss model. Acetate concentrations were determined with a Dionex Ion Chromatagraph (Model AI-450; Dionex Co., San Francisco, CA) equipped with a pulse electrochemical detector (Model DX300) using a 4-mm Ionpac AS10 column. Appropriate calibration curves were determined for each set of acetate samples.

Plasmid Stability and Expression Studies in Suspended Culture

Batch Reactor Studies

Experiments using the original plasmid host *B. cepacia* $PR1_{31}$ (TOM_{31c}) and the transconjugant *B. cepacia* 17616 (TOM_{31c}) growing on nonselective acetate-HCMM2 medium were carried out to compare levels of TOM expression, acetate growth characteristics, and TOM_{31c} stability between the two strains during batch growth. The batch reactors used were custom made from 1000-mL graduated glass beakers to provide a 500-mL total liquid volume. Reactors were continuously stirred at a rate of approximately 350 rpm and were aerated with 0.2 m filtered air at a rate of approximately 300 mL/min. The pH was monitored during each experiment and was found to range from 7.0 and 7.4.

A series of batch growth studies using acetate concentrations ranging from 2 to 20 m*M* were carried out. Acetate growth characteristics were determined by monitoring acetate and biomass concentrations over the duration of complete batch growth. Plasmid TOM_{31c} expression and activity were determined periodically during each batch experiment using the TFMP-suspended culture assay and 24-h overnight batch TCE disappearance assays.

Total, plasmid-free, and plasmid-bearing cell concentrations were determined periodically throughout each batch experiment to study the loss of plasmid TOM_{31c} in the batch 17616 (TOM_{31c}) cultures. Total cell numbers were determined by dilution plating on LBG agar plates. Ratios of plasmid-free to total cell counts and plasmid-bearing to total cell counts were determined using the plasmid TOM_{31c}-selective direct-colony transfer method (PSDCT method) described in what follows.

Continuous Culture Studies

All continuous culture experiments used custom-made chemostats with 500-mL working liquid volume. The chemostats were continuously stirred at a rate of approximately 350 rpm and were aerated with 0.2 μm of filtered air at a rate of approximately 300 mL/min. Chemostats were changed every 7 days to reduce the effects of wall growth. Changes in pH during each continuous culture experiment were found to be insignificant with an average pH of 7.1. Acetate-HCMM2-fed chemostat studies were carried out to determine the stability and activity of TOM_{31c} in host 17616 during continuous culture. Chemostats were run at dilution rates ranging from 0.05 to 0.19 h^{-1}. Steady-state TOM specific enzyme activities were determined using the suspended culture TFMP assay to determine if TOM expression was a function of growth rate. Plasmid-bearing, plasmid-free, and total cell counts were periodically determined at three of the different dilution rates using the PSDCT method to monitor plasmid loss during continuous growth and to determine if plasmid loss was a function of growth rate.

A series of TOM-selective, phenol-fed chemostat studies were performed to determine if selective phenol growth could be used to either stabilize or select TOM_{31c}-bearing cells in continuous culture. Phenol concentrations were determined using a modified colorimetric phenol assay presented by Folsom et al. (1990).

Analytical Methods and Protocols

Protein Assay

Protein content of all strains used was determined using the enhanced BA protein assay (Pierce Co.) using a Milton-Roy Spectronic 601 Photospectrometer. Cell number versus protein content, as well as A_{600} versus protein content calibration curves were determined for each specific microbial strain harvested from each growth medium. The protein assay was used in all of the TFMP assays, TCE disappearance assays, and suspended culture studies as a measure of biomass concentration.

TOM_{31c}-Selective Direct Colony Transfer Method (PSDCT)

The PSDCT method was used to determine total cell counts and the fraction (0.01 to 1.0) of plasmid-bearing and plasmid-free cells in a given culture (Sharp, 1995). The method involved the transfer of colonies grown on nonselective medium (LBG) to selective medium (phenol and phenol Km). The transferred colonies were then checked for both growth on the selective medium and TFMP activity (TOM activity). The PSDCT method determined the presence of TOM by indicating both the presence of the plasmid-borne kanamycin resistance (Tn5 transposon) and the expression of TOM and C230 (TFMP colony assay). In addition, the cells' ability to grow on phenol is a direct result of the presence of plasmid TOM_{31c}, giving a third indication of the presence and activity of TOM_{31c}.

MATHEMATICAL MODELS

Single substrate Michaelis–Menten kinetics obtained from sets of batch disappearance assays were used to model TCE

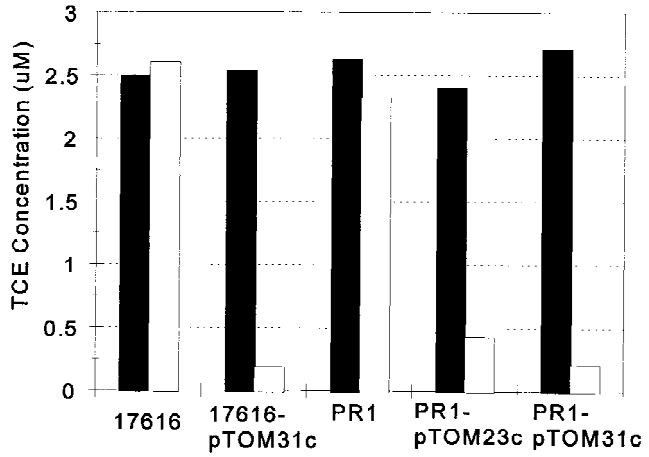

Figure 2. TCE removal by TOM_{31c}-free and TOM_{31c}-bearing 17616 and $PR1_{31}$ strains. Solid bars indicate the initial TCE concentration; empty bars indicate the TCE concentration after 24 h of shaking/incubation at 25°C.

biodegradation by *B. cepacia* 17616 (TOM_{31c}). Andrews inhibition kinetics [Andrews et al., 1968; see eq. (1)] were used to model acetate growth of both the plasmid-bearing and plasmid-free strains of 17616. Acetate studies were carried out to determine if the TOM_{31c} caused a detectable metabolic demand affecting the growth of 17616:

$$\mu = \frac{\mu_{max} S}{\left(K_s + S + \frac{S^2}{K_i} \right)} \quad (1)$$

A model for plasmid loss in continuous growth cultures was presented by Bailey and Ollis (1986). A mass balance on a plasmid-bearing population in a chemostat accounts for the rate of plasmid-bearing cell growth (μX^+), the rate of segregational plasmid loss ($p\mu X^+$), and the rate of cell mass leaving the continuous flow reactor via the effluent ($-DX^+$). A similar complementary balance on the plasmid-free cell population (X^-) can also be written. Both balances can be applied to a system where plasmid maintenance does not affect cell growth rate ($\mu^+ = \mu^- = \mu$). In this system, the total biomass concentration is the sum of the plasmid-bearing and plasmid-free populations in the system ($X = X^- + X^+$). For this system, Bailey and Ollis illustrate that the total biomass concentration (X) will have a nonzero steady-state value when the dilution rate is equal to the growth rate, $D = \mu$. In a steady-state chemostat system, the value for plasmid-bearing cell concentration (X^+) at time t is represented by eq. (2):

$$\ln X^+(t) = \ln X^+(0) - p\mu t \quad (2)$$

Each data set was correlated as per eq. (2), where the slope of a linear regression on $\ln X^+$ versus time would yield ($p \cdot \mu$). With the assumption that growth rate is equal to dilution rate ($\mu = D$) at steady state, an estimate of the probability of plasmid loss (p) was made.

RESULTS

Growth and TCE Disappearance Studies

Overnight (24-h) TCE disappearance and TFMP specific activity assays showed that TOM_{31c} expression in the transconjugant is equivalent to that of the TOM_{31c} in its original host (Fig. 2). In addition, the plasmid-free strains demonstrated no TCE degradative ability nor any TFMP activity. Results from a series of batch TCE disappearance assays show that specific TCE degrading activity of transconjugant 17616 (TOM_{31c}) follows simple single-substrate Michaelis–Menten saturation kinetics. Figure 3 shows that the saturation model correlated well with the kinetic data collected. From this correlation, the maximum TCE specific activity (V_{max}) was found to be 9.7 nmol TCE/min · mg protein and the corresponding half-saturation (K_m) constant was 5.4 μM TCE.

Batch acetate growth studies show that both the plasmid-bearing and plasmid-free 17616 strains follow Andrews inhibition kinetics when grown on acetate (Fig. 4). Figure 4 illustrates that there is no significant difference between the growth kinetics of 17616 and 17616 (TOM_{31}). Resulting acetate growth kinetic model parameters for each strain were $\mu_{max} = 0.49\ h^{-1}$, $K_s = 3.5$ mM acetate, and $K_i = 9.4$ mM acetate. Using these parameters, the highest obtainable inhibited growth rates for both the plasmid-bearing and plasmid-free cultures was found to be $0.22\ h^{-1}$.

Suspended Culture Plasmid Stability and Expression Results

Batch

Batch reactor plasmid loss studies showed that there was no measurable plasmid loss during batch growth of either the

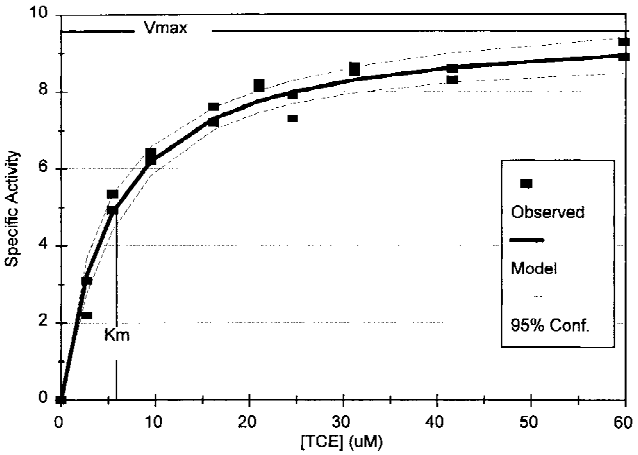

Figure 3. Monod kinetics for TCE mineralization by 17616 (TOM_{31c}), where $V_{max} = 9.7$ nmol TCE/mg protein · min and $K_m = 5.4$ μM TCE. Experiment was carried out in aerated HCMM2 medium at a pH of 7.2 and a temperature of ~25°C. Nonlinear curve fit (solid line) of $R^2 = 0.94$ and 95% confidence intervals (dashed lines) are shown.

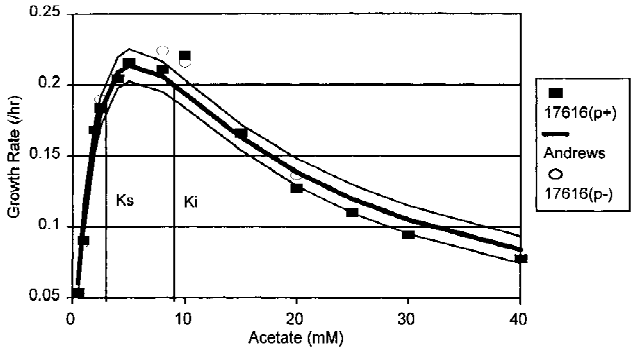

Figure 4. Andrews substrate inhibition growth kinetics for 17616 (TOM_{31c}) growing on nonselective. noncompetitive acetate medium, where $\mu_{max} = 0.49$/h, $K_s = 3.5$ m*M* acetate, and $K_i = 9.1$ m*M* acetate. Andrews model fit (solid line) with $R^2 = 0.956$ and 95% confidence intervals (dashed lines) shown.

17616 (TOM_{31c}) or $PR1_{31}$ (TOM_{31c}). This could be interpreted in two ways: (1) plasmid loss was not occurring in batch growth; or (2) the PSDCT method was unable to measure the plasmid loss in the finite number of cell divisions represented in the batch growth studies. These results suggest that plasmid loss in 17616 (TOM_{31c}) would be best studied under continuous culture conditions so that significant plasmid loss and/or loss of phenotype could be measured over many generations and under steady-state growth conditions.

Chemostat

Results from the nonselective continuous culture experiments showed that, after an initial lag phase (~10 generations), there was a significant loss of TOM_{31c} in 17616 (TOM_{31c}) cultures at all three growth rates. Figure 5 shows a plot of plasmid-bearing cell fractions and the total and true TFMP specific activities of the continuous cultures at three different dilution rates. The initial lag in measured plasmid loss was attributed to both the lack of sensitivity in the PSDCT method and the high kanamycin concentration (80 μg/mL) used to insure a pure plasmid-bearing culture in the initial chemostat reactor volume. It is believed that the high kanamycin concentration in the initial reactor volume resulted in the killing or inactivation of plasmid-free cells, thus not allowing them to persist in the reactor at the initial stages of each experiment.

Figure 5 shows the different magnitudes of TFMP specific activity for each of the three growth rates for which plasmid loss was measured. It should be noted that all of the colonies that did grow on phenol also proved to be kanamycin resistant and TFMP positive. In addition, all of the colonies that did not grow on phenol tested TFMP negative and were not resistant to kanamycin. These results indicate that, when one plasmid-borne phenotype was lost, all of the tested phenotypes were lost, suggesting segregational plasmid instability. Figure 6 shows the linear relationship between growth rate and the true TFMP specific activity of the continuous cultures. Plasmid-loss factors (p) were determined at three different growth rates ($\mu = 0.065$, 0.10, and 0.17) using eq. (2). These results are summarized in Table II.

Although a plasmid-loss factor was not determined for substrates other than acetate, significant plasmid loss was also noted in both nonselective LBG- and phthalate-fed continuous cultures. No plasmid loss was measured in selective phenol continuous cultures of 17616 (TOM_{31c}). In addition, no measurable plasmid loss was observed in any nonselective parallel experiments run with the original plasmid host $PR1_{31}$ (TOM_{31c}).

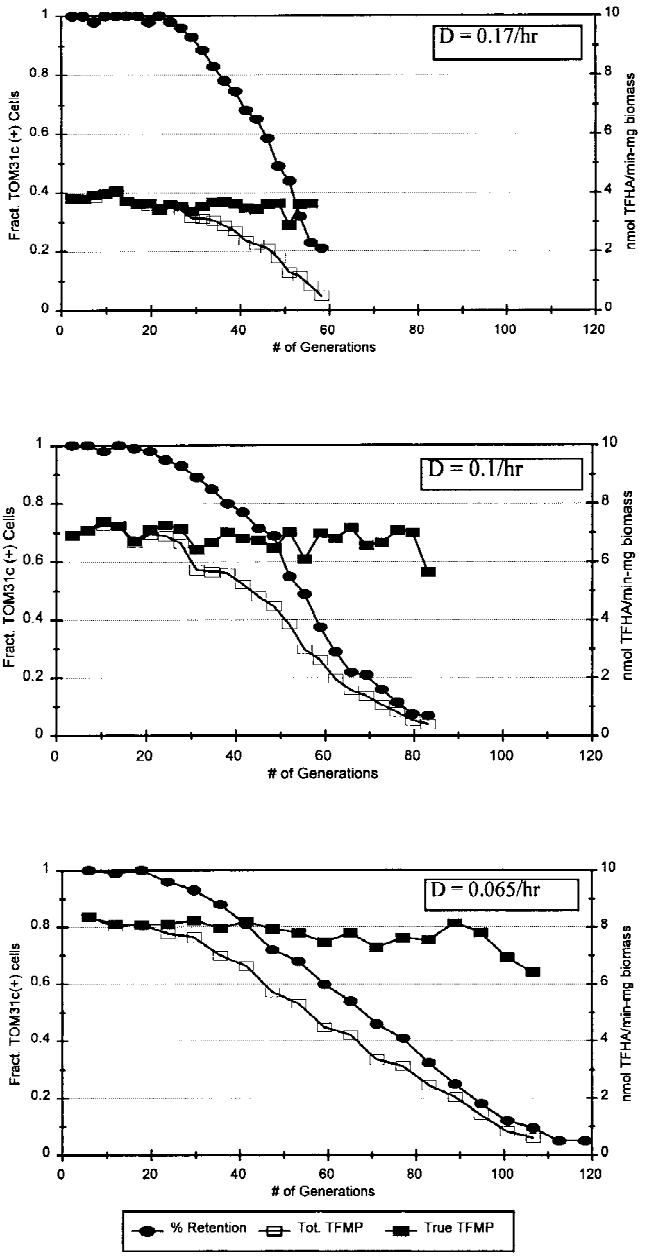

Figure 5. Plasmid-bearing cell fractions, total TFMP activities, and true TFMP activities versus number of generations for acetate-fed continuous 17616 (TOM_{31c}) cultures, where dilution rates (D) = 0.17, 0.1, and 0.065/h. Note the lag in measurable plasmid loss and the relative stability of true TFMP activities.

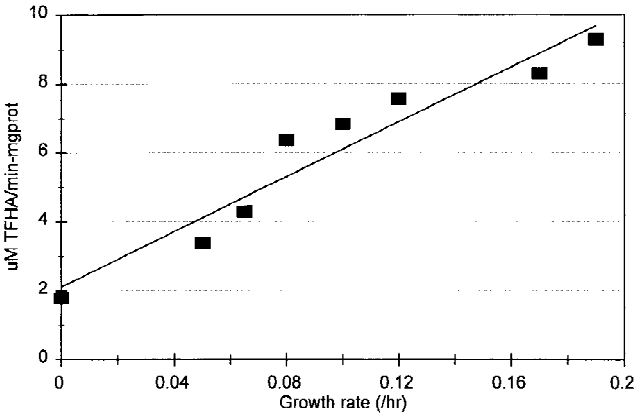

Figure 6. Linear relationship between growth rate and TOM_{31c} specific enzyme activity, where slope, $m = -27.8$, and y-axis intercept, $b = 2.1$ μmol TFHA/mg protein · min ($R^2 = 0.96$).

DISCUSSION

Results from the batch reactor studies indicate that *B. cepacia* 17616 harbored TOM_{31c} and constitutively expressed the TOM pathway. This is evident from results of TCE disappearance assays (Figs. 2 and 3) and TFMP assays. The TCE mineralization rates for 17616 (TOM_{31c}) shown in Figure 3 are equivalent to or slightly higher than those found for the original plasmid host *B. cepacia* G4 under similar growth conditions (Folsom et al., 1990). These results indicate that the 17616 (TOM_{31c}) transconjugant has successfully incorporated the plasmid and expressed the plasmid-borne TOM pathway at levels equivalent to those of the original plasmid host.

Results from acetate growth kinetics for both the plasmid-bearing and plasmid-free 17616 cultures (Fig. 4) show that plasmid maintenance had little or no significant metabolic demand on the host and did not result in a growth rate advantage for the plasmid-free cells. If maintenance and expression of the plasmid were to have a significant metabolic demand on 17616 (TOM_{31c}) cells ($\mu^+ < \mu^-$), the plasmid-free cells would have a growth rate advantage and would eventually overtake a culture, resulting in an inevitable loss in the desired TCE degradative phenotype. Such a loss would lead to inadequate TCE degradation efficiency and obvious process control problems.

Results from continuous culture experiments show that expression and subsequent activity of TOM_{31c}-encoded enzymes in 17616 is a linear function of growth rate, which can be described by eq. (3):

$$\text{True TFMP specific activity (nmol TFHA/mg protein} \cdot \text{min)} = 2.1 + 2382 \cdot [\mu(\text{min}^{-1})] \quad (3)$$

Figure 6 demonstrates the linear relationship between TOM specific activity and growth rate. It can be seen that, at zero growth rate, resting cells had a residual specific activity of 2.1 μmol TFHA/mg protein · min. The residual activity illustrates the continuous expression of TOM_{31c} under extremely low nutrient conditions. Because the maximum specific activity of TOM_{31c} changes with growth rate, so should the maximum TCE degradation specific activity (V_{max}). The effect of growth rate on specific activity indicates that, to attain an efficient TCE degradative TOM_{31c}-bearing culture, whether it be in a reactor or an in situ remediation system, there must be ample growth substrate and nutrients available. However, modest rates of TCE degradation can be achieved at minimal growth, as is illustrated by the residual activity of the resting cells.

Continuous culture plasmid-loss shown in Figure 5 illustrates that plasmid loss is a function of growth rate. The dichotomy between the effects of growth rate on TOM_{31c} expression (as measured by enzyme specific activity), and the effects of growth rate on plasmid loss demonstrates the need to maintain a *B. cepacia* 17616–TOM_{31c} culture at a relatively low growth rate to balance plasmid loss and TCE degradative activity. In addition, the apparent plasmid loss in continuous culture indicates that some form of selection for plasmid-bearing cells must be applied to the culture periodically to maintain a dominant and active TCE degrading population. Bias toward the plasmid-bearing population could be achieved through either antibiotic selection pressures using periodic doses of kanamycin to kill the plasmid-free population or the intermittent use of phenol or toluene as growth substrates, which only the plasmid-bearing cells can utilize.

Use of kanamycin is not economically feasible, but the intermittent use of selective carbon sources could be effective, especially in continuous culture reactors and high rate biofilters. Yet, only periodic use of selective carbon sources would be feasible, due to interactions with the plasmid-selective carbon sources and TCE, which result in competitive inhibition of TCE oxidation (Folsom et al., 1990).

Table II. Results from continuous culture plasmid retention and activity experiments: Using 17616 (TOM_{31c}) grown under nonselective conditions (acetate-HCMM2) and the plasmid loss model proposed by Ollis (1982).

Growth rate/ dilution rate (h^{-1})	Initial biomass X_o (cells/mL)	Plasmid loss factor (p)	True TFMP (spec. act.)	Model fit (R^2)
0.065	3.15×10^8	0.027	4.1	0.94
0.1	4.55×10^7	0.031	6.9	0.97
0.17	1.02×10^6	0.0335	8.25	0.95

Results from additional continuous culture experiments using other nonselective growth substrates (LBG and phthalate) also exhibited considerable plasmid loss (Sharp, 1995), indicating that plasmid loss is not an artifact of acetate growth but a consequence of either continuous culture, which has been suggested by Dwilvedi et al. (1982) and Primrose et al. (1984), or a process inherent in plasmid/host systems such as the one described here. A method for determining whether or not the plasmid instability described in this study is an artifact of continuous culture is to perform the same types of studies on biofilm cultures of the transconjugant 17616 (TOM_{31c}). This subject is addressed in a subsequent article (Sharp et al., 1997).

Our results show that, even though a plasmid may be initially incorporated into a desired population, its maintenance and activity may incur instabilities and inconsistencies depending on growth rate, selection parameters, and the relationship/interactions between the specific plasmid/host system.

CONCLUSIONS

The incorporation and expression of the TOM pathway in the transconjugant *Burkholderia cepacia* 17616 (TOM_{31c}) was demonstrated. *B. cepacia* 17616 (TOM_{31c}) was capable of degrading TCE at rates equivalent to those of the original TOM host (the phenol induced *B. cepacia* G4). Enzyme activity encoded by TOM_{31c} was found to be a linear function of growth rate, with a basal activity in the resting cells that results from the continuous, noninduced expression of TOM_{31c}. Degradative activity was maintained in plasmid-bearing cells during long-term growth in continuous suspended cultures.

Plasmid loss in 17616 (TOM_{31c}) determined using a continuous suspended culture mathematical model was considerable, resulting in an order-of-magnitude decrease in plasmid-bearing cells over 60 to 120 generations under nonselective growth conditions. This degree of plasmid loss would result in the critical loss of the TCE degradative phenotype in the culture, which would have a profoundly negative effect on the performance of TCE degrading bioremediation technology. Results show a need for plasmid selection and/or process control methods aimed at reducing plasmid loss while enhancing degradative activity. Such methods could lead to the long-term maintenance of an effective TCE degrading population of 17616 (TOM_{31c}) to be used in bioremediation technologies. Our studies illustrate a need to examine plasmid loss in environmentally relevant plasmid/host systems to determine the plasmid/host interactions that affect the performance and efficiency of bioremediation technologies. In addition, the study of these plasmid/host interactions in biofilm cultures is also suggested, due to the possibility of greater plasmid stability among biofilm populations and the abundance of biofilm processes associated with both in situ and reactor-based bioremediation technologies.

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NOMENCLATURE

μ	specific growth rate ($time^{-1}$)
μ^+	specific growth rate of plasmid-bearing cells ($time^{-1}$)
μ^-	specific growth rate of plasmid-free cells ($time^{-1}$)
V_{max}	Michaelis–Menten maximum specific activity ($time^{-1}$)
μ_{max}	maximum specific growth rate ($time^{-1}$)
K_s	Monod half-saturation constant (m*M* or mg/L)
K_m	Michaelis–Menten half-saturation constant (m*M* or mg/L)
K_i	Andrews substrate inhibition half-saturation constant (m*M* or mg/L)
S	concentration of growth substrate (m*M* or mg/L)
X^+	plasmid-bearing cell concentration (cells/mL)
X^-	plasmid-free cell concentration (cells/mL)
t	time (h or days)
p	plasmid loss factor
D	dilution rate ($time^{-1}$)

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