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

Robert R. Sharp,[1] James D. Bryers,[2] Warren G. Jones[2]

[1]Department of Environmental Engineering, Manhattan College, Riverdale, New York 10475; telephone: 718-862-7169; fax: 718-862-8018; e-mail:RSHARP@manhattan.EDU
[2]Center For Biofilm Engineering, Montana State University, Bozeman, Montana 59717

Received 31 January 1997; accepted 3 December 1997

Abstract: The activity and stability of the TCE degradative plasmid TOM_{31c} in the transconjugant host *Burkholderia cepacia* 17616 was studied in selective and nonselective biofilm cultures. The activity of plasmid TOM_{31c} in biofilm cultures was measured by both TCE degradative studies and the expression of the Tom pathway. Plasmid loss was measured using continuous flow, rotating annular biofilm reactors, and various analytical and microbiological techniques. The probability of plasmid loss in the biofilm cultures was determined using a non-steady-state biofilm plasmid loss model that was derived from a simple mass balance, incorporating results from biofilm growth and plasmid loss studies. The plasmid loss model also utilized Andrew's inhibition growth kinetics and a biofilm detachment term.

Results from these biofilm studies were compared to similar studies performed on suspended cultures of *Burkholderia cepacia* 17616-TOM_{31c} to determine if biofilm growth has a significant effect on either plasmid retention or Tom pathway expression (i.e., TCE degradation rates). Results show that the activity and expression of the Tom pathway measured in biofilm cultures was significantly less than that found in suspended cultures at comparable growth rates. The data obtained from these studies fit the plasmid loss model well, providing plasmid loss probability factors for biofilm cultures that were equivalent to those previously found for suspended cultures. The probability of plasmid loss in the *B. cepacia* 17616-TOM_{31c} biofilm cultures was equivalent to those found in the suspended cultures. The results indicate that biofilm growth neither helps nor hinders plasmid stability. In both the suspended and the biofilm cultures, plasmid retention and expression could be maintained using selective growth substrates and/or an appropriate plasmid-selective antibiotic. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **59**: 318–327, 1998.
Keywords: plasmid; retention; TCE; biofilm; segregational stability; activity

INTRODUCTION

Bacteria and their exopolymer secretions associated with an interface, are known as biofilms (Characklis and Marshall, 1990). Biofilms have been used to remove pollutants from wastewater for hundreds of years, and thus are the fundamental basis of many traditional wastewater treatment processes. More recently, biofilms have been used in bioremediation technologies to degrade xenobiotics found in industrial effluents, groundwaters, and contaminated soils. One use for biofilm-based remediation technologies is the removal of trichloroethylene (TCE) from groundwaters and soils. TCE, a solvent used in the dry cleaning industry, is a ubiquitous and recalcitrant groundwater contaminant and is an US-EPA priority pollutant.

A number of biological methods have been developed to degrade TCE. The majority of these methods involve aerobic bacterial isolates and microbial consortia capable of cometabolic mineralization or degradation of TCE (Finette et al., 1984; Folsom and Chapman, 1991; Folsom et al., 1990; Gibson et al., 1982; Kaphammer et al., 1990; Nelson et al., 1987; Shields and Reagin, 1992; Tsien et al., 1989; Vandenbergh and Kunka, 1988; Wackett and Gibson, 1988). One such bacterial species is *Burkholderia cepacia* $PR1_{31c}$ (TOM_{31c}) (formerly classified as *Pseudomonas cepacia*), which is a Tn5 transposon mutant of the environmental isolate *B. cepacia* G4 (Nelson et al., 1987; Shields et al., 1995). *Burkholderia cepacia* $PR1_{31c}$ (TOM_{31c}) carries its constitutive, cometabolic TCE degradative pathway on the plasmid TOM_{31c}. TOM_{31c} is a transmissible plasmid that includes gene sequences which encode for all of the proteins needed for constitutive mineralization of TCE via a cometabolic pathway, the newly defined Tom (toluene orthomonooxygenase) pathway (Shields et al., 1989). These proteins include toluene ortho-monoxygenase (TomA) and catechol 2,3 dioxygenase (C230 or TomB). Plasmid TOM_{31c} also includes the Tn5 transposon insertion containing a kanamycin resistance marker. Involvement of the Tom pathway in the aerobic mineralization of TCE has been definitively characterized in the literature (Shields et al., 1995; Shields and Reagin, 1992). Plasmid TOM_{31c} could have a number of important applications in the field of bioremediation. For example, TOM_{31c} could be transferred into indigenous bacterial populations to increase the resident TCE degradative activity, or it could be applied to highly effective reactor-based remediation technologies. Research using cultures other than PR1 has indeed taken

Correspondence to: Robert Sharp

© 1998 John Wiley & Sons, Inc. CCC 0006-3592/98/030318–10

place. Speital and Leonard (1992) used a sequencing biofilm reactor to treat TCE. Finnell et al. (1993) used a methanotrophic attached-film, expanded bed reactor to degrade TCE at a maximum rate of 0.8 mg TCE/g VSS-day. Strandberg et al. (1989) also used a methanotrophic consortium to degrade trichloroethylene and trans-1,2-dichloroethylene in a fixed film reactor. Freedman and Gosset (1989) and Vogel and McCarty (1985) have experimented with anaerobic cultures to perform low rate, reductive dechlorination of TCE. Yet, most of these processes involve slow growing cultures that often do not readily produce a biofilm. In addition, a number of these cultures produce highly toxic intermediates and byproducts such as vinyl chloride (Alvarez-Cohen and McCarty, 1991a). For these reasons, the application of many TCE degrading cultures in either reactor-based or in-situ bioremediation would be impractical for full-scale use.

Attempts to utilize *B. cepacia* PR1 in biofilm reactors to treat vapor phase TCE had limited success during short-term operation (Shields et al., 1993). However, during long-term treatment the biofilm cultures readily lost their ability to degrade TCE at a significant rate (Sharp et al., 1997; Sharp, 1995; Shields et al., 1996). This apparent loss of TCE degrading ability was noted by decreased efficiency, lack of biomass accumulation, and eventual failure to degrade TCE. This failure may be attributed to a number of possible phenomena, including chemical toxicity, plasmid instability, lack of biofilm production and/or cell injury (Sharp, 1995). To exploit more effectively the unique degradative capabilities of TOM_{31c}, and other environmentally relevant plasmids in a biofilm-based remediation process, one must first understand the behavior and characteristics of the plasmid-bearing populations in biofilm culture.

The key to developing an efficient, reliable TCE degrading biofilm system is finding a suitable host to harbor and express a degradative plasmid such as TOM_{31c}. Characteristics for a suitable host include: an ability to readily attach to a surface and produce a biofilm, a resistance to TCE-related injury and/or toxicity, and an ability to retain and express the plasmid-borne pathway during long-term growth. Microorganisms carrying plasmids are susceptible to both segregational and structural instability, both of which can lead to the permanent loss of a desired plasmid-borne phenotype or genotype. Factors affecting plasmid stability include: immobilization and attachment (Dykhuizen and Hartl, 1983; Huang et al., 1993; Inloes et al., 1983; Kumar et al., 1991) nutrient availability and limitations (Huang et al., 1994; Jones and Melling, 1984; Jones et al., 1980; Sayadi et al., 1989), plasmid copy number (Jones et al., 1980; Peretti and Bailey, 1987; Sayadi et al., 1989; Uhlin and Nordstrom, 1987), growth rate (Seo and Bailey, 1985; Stewart and Carlson, 1986; Taxis du Poët et al., 1987), and selection (Lauffenburger, 1987; Tiedje et al., 1989; Wood et al., 1990). Although most research on plasmid stability has focused on suspended cell cultures (Dwilvedi et al., 1982; Dykenhuizen and Hartl, 1983; Ensley, 1986; Grandi et al., 1981; Kadam et al., 1987; Noack et al., 1982; Primrose et al., 1984; Roth et al., 1980; Roth and Noack, 1982; Sherrat, 1982; Summers, 1991), the majority of technologies in the area of bioremediation involve immobilized or biofilm cultures. Compared to suspended cultures, biofilm and immobilized cells may experience increased plasmid stability for two reasons: (1) the proximity of biofilm cells to one another may improve intracellular plasmid transfer mechanisms; and (2) the mass transfer limitations, inherent in immobilized and biofilm systems, may lower growth rates inside the biofilm.

Inloes et al. (1983) reported increased plasmid stability in *E. coli* cultures immobilized in a non-selective hollow fiber system. Taxis du Poët et al. (1987) reported increased stability in plasmid pTG201 in carrageenan encapsulated *E. coli* cultures. Both of these studies attribute the increased plasmid stability to: (a) mass transfer limitations creating slower biofilm cell growth rates, (b) the possibility of higher rates of plasmid transfer due to cell-to-cell proximity, and (c) the reduction of structural instabilities in the recombinant cells. Similar results using different plasmid/host systems and different immobilization techniques have been reported by other researchers. (Nasri et al., 1987; Kumar and Schugerl, 1990; Sayadi et al., 1989).

In biofilm cultures, a number of researchers have found significant plasmid transfer may reduce the probability of plasmid loss (Levin et al., 1979; Saye et al., 1987; Stewart and Carlson, 1986). In contrast, Huang et al. (1993) found decreased stability of the industrial plasmid pMJR1750 in *E. coli* DH5 when grown as a biofilm. The decrease in plasmid stability was attributed to plasmid copy number differences between suspended and biofilm cultures and the metabolic demand of the production of the biofilm extracellular matrix polymers, the production of which compete for plasmid maintenance/replication energy.

To utilize certain recombinant plasmids in environmental remediation systems, specific plasmid/host interactions must be well understood. In addition, the use of plasmid recombinant microorganisms, such as *B. cepacia* $PR1_{31c}$ (TOM_{31c}), in open systems mandates research into the fundamental plasmid/host processes that influence plasmid retention, stability, expression, and transfer within a biofilm system. This article reports on the laboratory evaluation of the retention and expression of the TCE degrading plasmid TOM_{31c} in the host *B. cepacia* 17616 in biofilm cultures and includes the use of a modified segregational plasmid loss mathematical model, which employs kinetics for both the growth and detachment of biofilm cells. In addition, this article compares previously reported plasmid-loss results found for suspended cultures of B. cepacia 17616 TOM_{31c} with the biofilm culture results described here (Sharp et al., 1997).

MATERIALS AND METHODS

Plasmid/Host System

Transconjugant *B. cepacia* 17616 (TOM_{31c}) was used in all suspended and biofilm experiments to evaluate plasmid

maintenance, expression, and stability in biofilm cultures. Plasmid TOM_{31c} presence in a cell was determined using either its kanamycin resistance, which is associated with the plasmid-borne Tn5 transposon insertion, or its ability to utilize various hydrocarbons as substrates (phenol). *Burkholderia cepacia* 17616 was chosen as the host for TOM_{31c} because the bacteria has been well characterized (Cheng and Lessie, 1994), and is known to attach readily to surfaces and produce significant amounts of biofilm (Murgel et al., 1991). Plasmid-bearing strains of transconjugant host *B. cepacia* 17616 and the original host *B. cepacia* PR1 were supplied by Shields CEDB, Univ. West Florida, Pensacola, FL. Cultures of the original plasmid host, *Burkholderia cepacia* PRI_{31c} (TOM_{31c}), were used in a number of studies either as controls or for comparison to *B. cepacia* 17616 (TOM_{31c}) cultures. Glycerol/peptone frozen cultures (−70°C) of plasmid-free and plasmid-bearing cultures were maintained and used for starter cultures in all of the experiments. Selective phenol-kanamycin agar plates and slants of plasmid-bearing cultures were maintained and re-streaked as needed. All experiments were inoculated with a culture of TOM_{31c} bearing cells harvested from highly selective starter cultures. In addition, all continuous flow experiments, selective and non-selective, were run with 80 μg/mL of kanamycin in the initial reactor volume to insure that each experiment started with a pure plasmid-bearing culture.

Media

Three different media were used in this research: (1) a ''rich'' general growth media, Luria Broth Glucose (LBG—10 g/L Tryptone, 5 g/L yeast extract, 5 g/L NaCl, 1 g/L glucose); (2) a non-selective media, hydrocarbon minimal media (HCMM2) amended with the non-selective carbon source, acetate (0.5–40 m*M* sodium acetate); and (3) a selective media, HCMM2 amended with phenol (2 m*M*) and kanamycin (50 μg/mL). Acetate was chosen as a non-selective growth substrate because *B. cepacia* 17616 grows well on acetate, and acetate is easily analyzed using ion chromatography and does not competitively inhibit the TCE degradative pathway. All non-selective agar plates were made with 15 g/L bacto agar (Difco, Detroit, MI), while all plasmid-selective plates using phenol were made with low carbon Noble Agar (Difco) to insure phenol was the sole growth substrate.

TOM_{31c} Specific Activities

The m-trifluoromethylphenol (TFMP) assay was used to indicate the expression of the Tom pathway by measuring the activity of the TOM_{31c} encoded enzymes toluene ortho-mono-oxygenase (Tom) and catechol 2,3 dioxygenase (C230). The TFMP assay was carried out as per Sharp et al., (1997). The TFMP assay allows for the quantification of the specific activity (mg TFHA produced/mg-biomass-minute) of the Tom pathway. Where TFHA (7,7,7 TF2-hydroxy-6oxo-2,4-heptadienoic acid) is the product of TFMP once transformed by the Tom and C230 enzymes. The results from the TFMP assays are reported as either the specific Tom activity of the whole culture, referred to as the ''Total TFMP Activity,'' or as the specific Tom activity relative to the plasmid-bearing fraction only, referred to as the ''True TFMP Activity.''

Biofilm Culture Plasmid Stability and Activity Studies

Twenty-four hour batch TCE studies were performed on surface samples taken from biofilm cultures of *B. cepacia* $PR1_{31c}$ (TOM_{31c}). Biofilm cultures of *B. cepacia* 17616 (TOM_{31c}) were cultivated on selective media (2 m*M* phenol-HCMM2 media with 50 μg/mL kanamycin) in a rotating annular reactor, collected and placed in 50 mL crimp-top anaerobic serum bottles with 25 mL of HCMM2 media. Each bottle was aerated with 0.2 μm filtered oxygen for 2 min to assure an excess of oxygen, then sealed with Teflon™ coated septa. TCE was added using a gas-tight syringe at an approximate final liquid concentration of 2.8 μ*M*. Bottles were incubated upside down at 25°C, and shaken for 24 h. Initial and final TCE concentrations were taken to determine if the resuspended biofilm cultures of transconjugant *B. cepacia* 17616-TOM_{31c} were expressing the Tom pathway and capable of readily mineralizing TCE.

TFMP assays of resuspended, selective 17616 (TOM_{31c}) biofilm cultures were performed to quantify the specific activity of TOM. These activities were compared to those previously reported for suspended cultures of 17616 (TOM_{31c}) (Sharp et al., 1997).

Burkholderia cepacia 17616 (TOM_{31c}) biofilm cultures were grown on non-selective media to determine the stability and activity of TOM_{31c} in biofilm culture. These biofilm cultures were grown using a rotating annular reactor system shown in Figure 1. Table I shows the physical and operational characteristics of the two sets of annular reactor studies. The annular reactors were initially colonized under batch operation with a high kanamycin selection (80 μg/mL) to insure the initial biofilm was essentially all plasmid-bearing cells. No cells were added to the system after inoculation. Biofilm studies were carried out at two different acetate concentrations, 4.8 m*M* and 8.2 m*M*, respectively. Once inoculated and colonized, annular reactors were operated at a dilution rate of at least 1.0/h (about 5 times greater than the maximum growth rate of 17616) to minimize the contribution of suspended cell growth. Sampling the annular reactors involved removal of 1 of the 12 polycarbonate slides located flush to the inside of the outer drum of the annular reactor (see Fig. 1). A slide was removed every 2–3 d and was replaced with a sterile slide to maintain consistent flow characteristics throughout each experiment. Each experiment was run in triplicate and utilized 12 biofilm samples, one sample for each of the original 12 reactor

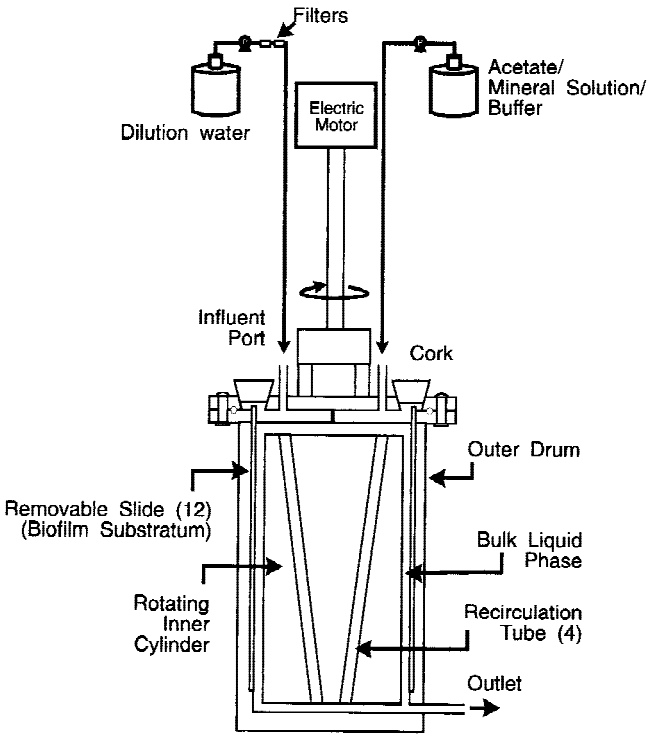

Figure 1. Diagram of the rotating annular reactor used to study plasmid/host interactions in biofilm cultures.

slides. Some of the experiments lasted as long as 30 d while others lasted only 15 d, due to either contamination or premature use of the 12 sample slides.

Protein and acetate concentrations of reactor effluent were determined periodically throughout each experiment. Total cell, plasmid-bearing cell [p(+)], and plasmid-free cell [p(−)] concentrations in both the biofilm and effluent samples were determined using the plasmid *tom*$_{31c}$ selective direct colony transfer method (PSDCT). Biofilm samples for the PSDCT method were harvested from the annual reactor slides using the method described by Srinivasan et al. True and total Tom specific activities of the biofilm samples were determined using the suspended culture TFMP assay.

Table I. Physical and operational characteristics of annular biofilm reactors.

Parameter/characteristic	Annular reactor
Reactor surface area (cm^2)	1900
Dilution rate (h^{-1})	1.0
Volume (mL)	600
Number of slides	12 (16 cm^2 each)
Flow rate (mL/min)	10
pH	7.4
Temperature (°C)	27 ± 1.5
Rotational speed (rpm)	50–60
Number of non-selective runs	6 (3 at each acetate concentration, 4.8 and 8.2 m*M*)
Number of selective runs	3 (each at a growth rate of ~0.2)

Analytical Methods and Protocols

Plasmid 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) of plasmid-bearing and plasmid-free cells in a given culture. The method involved the steps shown in Table II, and was performed in triplicate on all resuspended biofilm samples. Resuspended biofilm cells were also used in the 24 h batch TCE disappearance studies and in the TFMP specific activity assays.

Protein Assay

The protein content of all strains used was determined using the enhanced BA Protein Assay (Pierce Co., Rockford, IL) with a Milton Roy Spectronic 601 photo spectrometer. Cell number vs. protein content, as well as A600 vs. protein content calibration curves, were determined for each specific microbial stain harvested from each growth medium. The protein assay was used as a measure of total biomass in all of the TFMP assays, TCE disappearance assays, batch suspended cultures, and biofilm culture studies.

Acetate Analysis

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 4mm Ionpac AS10 column. Appropriate

Table II. The TOM selective direct colony transfer (PSDCT) method used to determine plasmid-bearing and plasmid-free cell numbers in biofilm cultures.[a]

Step	Procedure
1	Remove sample slide from annular reactor and replace with a sterile slide to maintain hydraulic flow regime.
2	Scrape and wash biofilm off of slide using 40 mLs of sterile HCMM2 media and a sterile Teflon spatula.
3	Homogenize 40 mL sample at medium speed for 5 min to separate cells and break up the EPS matrix.
4	Dilution plate sample on general growth LBG agar media and incubate for 2 d at 30°C to determine total cell counts. (Total viable cell counts)
5	Transfer 100 LBG (general growth) colonies directly to selective 2 m*M* phenol-kanamycin low carbon agar plates via a sterile toothpick.
6	Incubate selective plates for 2 d at 30°C and count all colonies. This number represents all of the colonies that have an active Tom pathway and still contain the Km resistance associated with pTOM$_{31c}$
7	Perform a TFMP colony assay on selective plates, and count the number of TFMP positive colonies. This count represents the plasmid bearing and pTOM active colonies. Only colonies that grew up on selective media and were TFMP positive were considered to be plasmid-bearing.[b]

[a]The PSDCT method was performed in triplicate to give three values for total cell counts, and plasmid-free and plasmid-bearing cell fractions for each sample.

[b]Every colony that grew on selective media was also found to be TFMP positive.

calibration curves were determined for each set of acetate samples.

Suspended Culture Studies

The suspended culture plasmid stability studies that are referred to in this article are described in detail by Sharp et al. (1997). These CFSTR studies were performed using the same substrates and similar substrate concentrations and growth rates as were used in this study. Plasmid loss in the CFSTR studies was determined using the PSDCT method. Acetate growth kinetics for both the plasmid-bearing and the plasmid-free strains of 17616 were determined from these continuous flow, suspended growth studies. All of the analytical techniques were the same as described here for the biofilm studies.

MATHEMATICAL MODELS

Plasmid loss in biofilm cultures was described using a modified version of a model presented by Huang et al. (1993); a system of equations based on mass balances of the plasmid-bearing [p(+)] and plasmid-free [p(−)] cells in the biofilm. The following assumptions and operating conditions were made to simplify the model:

1. No cells are introduced into the system after initial inoculation.
2. The system is operated at a high dilution rate (1.0 h^{-1}), thus any detached cells will have a short residence time (D > 0.2 h^{-1}) in the reactor. This operating condition, combined with the first condition, allows one to assume that the rates of growth of suspended (detached) cells within the system are negligible (Dilution rate, 1.0 h^{-1} > growth rate, 0.22 h^{-1} max).
3. Plasmid presence or loss in a population does not affect attachment or detachment rates of these cells.
4. The relative amount of plasmid-bearing cells and plasmid-free cells, detached from the biofilm is identical to the relative amounts of each population within the entire biofilm. This assumption tacitly implies that the two strains do not spatially stratify within the biofilm, thus their rates of detachment from the biofilm are assumed to be equal ($K_{det} = K_{det+} = K_{det-}$).

With these assumptions, the accumulation of plasmid-bearing cells and plasmid-free cells in a biofilm culture becomes a function of attached cell growth, plasmid loss, and biofilm detachment rate:

Plasmid-bearing biofilm cells:

$$dB^+/dt = \mu^+ B^+ - p\ \mu^+ B^+ - K_{det+} B^+ \quad (1)$$

Plasmid-free biofilm cells:

$$dB-/dt = \mu^- B^- + p\ \mu^- B^+ - K_{det-} B^- \quad (2)$$

Huang et al. (1993) solved this model for a system where the plasmid-bearing cells exhibited a different growth rate than the plasmid-free cells. Using a number of substitutions and dimensionless population fractions, Huang et al. derived a linear algebraic expression for the plasmid loss factor, p, as a function plasmid-bearing population densities, plasmid-free population densities, and their relative growth kinetic parameters.

For a system where there is no measurable growth rate differential between the plasmid-bearing and plasmid-free populations, the above simplifications cannot be made; Equations (1) and (2) must be solved numerically using finite difference methods. To directly solve either Equation (1) or (2), expressions for the biofilm detachment rate (K_{det}) and growth rate (μ) must be obtained.

TCE degradation kinetics for *B. cepacia* (17616-TOM_{31c}), along with the acetate growth kinetics of both the plasmid-bearing and the plasmid-free strains of 17616 were reported earlier (Sharp et al., 1997). Single substrate Michaelis-Menten saturation kinetics were used to model the TCE biodegradation by 17616 (TOM_{31c}). Andrews inhibition kinetics for 17616 acetate growth [Eq. (3)] were used to model acetate growth of both the plasmid-bearing and plasmid-free strains of 17616. Acetate growth kinetics were determined to evaluate whether or not the plasmid TOM_{31c} had a metabolic demand on the growth of 17616. The resultant kinetic expression [Eq. (3)] was used in the plasmid loss model.

$$\mu = \frac{\mu_{max} S}{\left(K_s + S + \frac{S^2}{K_i}\right)} \quad (3)$$

A kinetic expression used for detachment rate (K_{det}) of biofilm cells was developed by Camper and Jones to model the detachment of coliform biofilm cells in a rotating annular reactor. The model determines a biofilm detachment rate when the influent and effluent cell concentrations are known and when the dilution rate (D) and the specific area for biofilm formation (a) are constant.

$$K_{det} = 2\left(1/a \frac{(X_{bi} - X_{bi-1})}{(t_i - t_{i-1})}\right) - D/2(X_{bOi} + X_{bOi-1}) - X_{bi} - X_{bi-1} \quad (4)$$

The resulting expression for the probability of plasmid loss in biofilm cultures can be determined using the plasmid-free cell mass balance [Eq. (1)], where μ can be expressed by the Andrews substrate inhibition model [Eq. (3)] and K_{det} can be expressed by the biofilm detachment model [Eq. (4)]:

$$p = \frac{\frac{dB^-}{dt} - \mu^- + K_{det} B^-}{\mu B^+} \quad (5)$$

Plasmid loss in biofilm cultures was then compared to plasmid loss in suspended continuous cultures, which has been reported by Sharp et al. (1997) in a companion article. They utilized a steady-state suspended culture plasmid loss model presented by Bailey and Ollis, shown here as Equation (6).

$$\ln X^+(t) = \ln X^+(0) - p\ \mu\ t \quad (6)$$

RESULTS

Tom Specific Activity and TCE Disappearance Studies on Selective Cultures

Results from 24 h TCE disappearance studies show that suspended and biofilm cultures of both the transconjugant, *B. cepacia* 17616 (TOM_{31c}), and the original host, *B. cepacia* $PR1_{31c}$ (TOM_{31c}), removed over 90% of the initial 2.8 μM TCE. These results indicate that the transconjugant 17616 (TOM_{31c}) can maintain and express the TCE degradative pathway under selective growth (phenol) conditions in both suspended and biofilm cultures. Plasmid-free strains of 17616 were used as negative controls, and demonstrated no removal of TCE in the 24 h studies.

Figure 2 shows the true TFMP specific activities of both 17616 (TOM_{31c}) and $PR1_{31c}$ (TOM_{31c}) in suspended and biofilm cultures, and plasmid-free strains of both 17616 and PR1 in suspended culture. All suspended and biofilm samples shown in Figure 2 were harvested from highly selective reactors at an equivalent growth rate of approximately 0.2/h. These results indicate that the biofilm cultures have a significantly lower activity than the suspended cultures. The lower specific activity may be a result of either: (1) assay interference caused by the extracellular polysaccharide associated with the biofilm culture, a lower growth rate resulting from the time required to sample the biofilm, which may lead to down regulation; or (2) the influence of EPS which may have increased the amount of protein measured in the sample, thus lowering the true specific activity.

Biofilm Studies

Figure 3 shows the results from two biofilm studies indicating the dynamics of the total, plasmid-free [p(−)], and plasmid-bearing [p(+)] 17616 populations over time at growth rates of either 0.2/h (Fig. 3a) or 0.22/h (Fig. 3b).

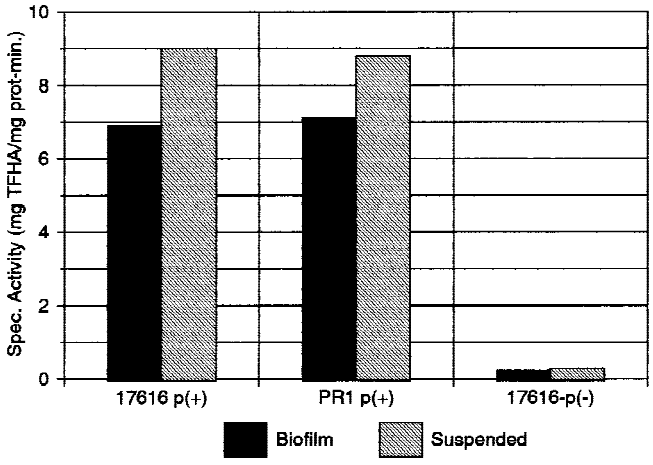

Figure 2. Plasmid TOM true specific activities as measured using the TFMP assay. Results are shown for the transconjugant 17616-TOM_{31c}, the original plasmid host PR1-TOM_{31c}, and a plasmid-free control harvested from both suspended and biofilm cultures.

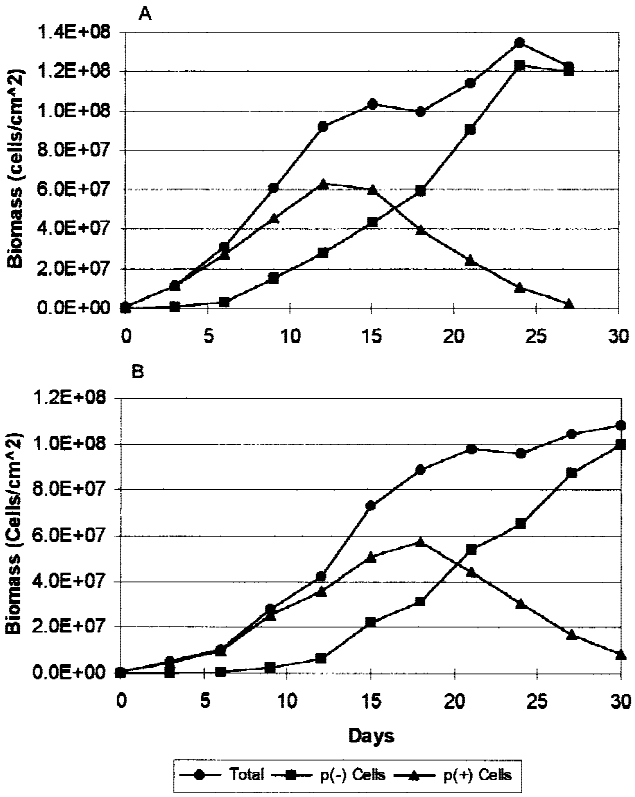

Figure 3. Population dynamics within the annular reactor biofilm cultures. Results show the change over time of the total, plasmid-bearing, plasmid-free cell populations in annular reactors operated at **(A)** 0.2/h which corresponds to an acetate concentration of 4.8 m*M*, and **(B)** 0.22/h which corresponds to an acetate concentration of 8.2 m*M*.

These average growth rates correspond to the average acetate concentrations of 4.8 m*M* and 8.2 m*M*, respectively, for these two runs (Sharp et al., 1997). As can be seen, the total number of cells continued to grow with increasing time, eventually leveling-off at a pseudo steady-state concentration. The number of p(+) cells initially increased with time as the accumulation rate was greater than the plasmid-loss rate, then decreased as steady state was reached. The p(−) populations experienced long initial log phases, then increased constantly until a pseudo-steady state was reached. The constant or steady increase in p(−) cells represents the dB/dt term found in Equation (5).

Figure 4 shows the p(+) fraction of cells and the total and true TOM activities for the same two experiments as biofilm growth approached a steady-state total cell density. These results demonstrate that as the plasmid was lost, i.e., the fraction of p(+) cells decreased, the total TOM specific activity of the total populations decreased. Yet, the remaining p(+) cell fraction maintained a relatively constant true TOM specific activity.

Figure 5 shows the fraction of plasmid-free cells in both the reactor effluent and the biofilm for each of the growth rates. The effluent cells represent only detached biofilm cells, because the dilution rate (1.0 h^{-1}) was much greater than the respective growth rates (0.2 and 0.22 h^{-1}). As can

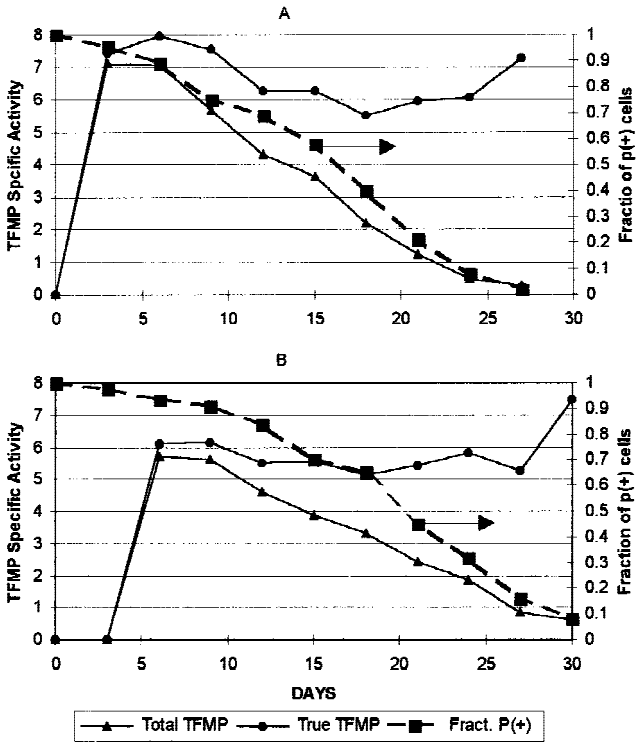

Figure 4. Results from annular reactor studies showing a decrease in plasmid-bearing cell fractions and total TFMP specific activities with relatively constant true TFMP specific activities for each of the two reactors. **(A)** Growth rate of 0.2/h^{-1}, and **(B)** Growth rate of 0.22 h^{-1}.

be seen, the same relative fractions of p(−) biofilm cells detached from the biofilm as there were growing in the actual biofilm. This continuity indicates that there was no preferential detachment of either cell type from the biofilm and support assumptions 3 and 4 which were made when developing the plasmid-loss model.

Using the unsteady-state modified version of the Huang et al. plasmid-loss model, plasmid-loss factors for the two sets of biofilm studies were determined. The model incorporated the Andrews inhibition kinetics coefficients for *B. cepacia* 17616 growth on acetate (Sharp et al., 1997) and the Camper and Jones (1997) biofilm detachment model.

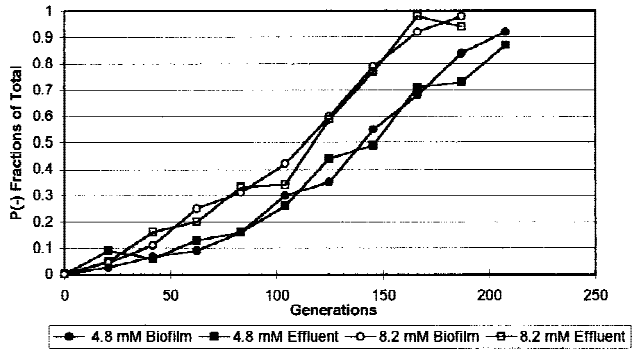

Figure 5. Comparison of plasmid-free cell fractions in both the effluent and biofilm samples over time for each of the annular reactor studies.

Detachment rates were determined from the cell concentrations and the specific characteristics of the annular reactors. Table III summarizes the average results from both sets of biofilm experiments and the biofilm plasmid-loss modeling effort.

DISCUSSION

Results from the 24 h TCE disappearance assays show that biofilm cultures of transconjugant *B. cepacia* 17616 (TOM_{31c}) can degrade TCE and express the Tom pathway at levels equivalent to those of the original host and to suspended cultures. Comparison of TFMP specific activities of 17616 (TOM_{31c}) in biofilm cultures vs. continuous flow suspended cultures show that the biofilm cultures have a lower true specific activity than the suspended cultures. Figure 6 shows a linear function of the suspended culture true TFMP specific activities vs. growth rate and the true TFMP specific activities for the respective biofilm cultures. It can be seen that the TFMP specific activities of the biofilm cultures are significantly lower than those found for suspended cultures at the same growth rate. These results indicate that although the transconjugant 17616 (TOM_{31c}) can successfully express the plasmid-borne Tom pathway in biofilm culture, this expression is less than that observed in suspended cultures.

There are a number of possible reasons for this decrease in specific activity in biofilm cultures including: (1) the metabolic priority to produce extracellular polysaccharide (the biofilm structural matrix) may decrease the activity or expression of the Tom pathway; (2) mass transfer limitations within the biofilm may result in a lower growth rate than expected from the Andrews inhibition kinetics, thus decreasing the Tom expression and activity; (3) effects of both the time and method used to sample and suspend the biofilm sample may allow for down regulation of the Tom pathway; (4) particles of extracellular polysaccharide (EPS) in the samples may interfere with either the conversion of TFMP to TFHA, or increase the apparent protein content in the sample, both would decrease the true TFMP specific activity. Mass transfer limitations were assumed to be to minimal due to: (1) the relative thinness of the biofilms (10–100 μm), (2) the completely mixed environment within the reactors, and (3) the high dilution rate at which the reactors were operated. Although maintenance energy cannot be ignored, the effects of down regulation during sample preparation and the effects of EPS particles are considered to be the most likely reasons for the decrease TFMP specific activities.

Results from the biofilm plasmid-loss experiments demonstrate that significant plasmid loss in 17616 (TOM_{31c}) also occurs in biofilm culture (Figs. 3, 4, and 5). Equation (5) was used to determine the plasmid-loss probability factor for the biofilm studies. This equation includes both the experimentally determined Andrews kinetic parameters [Eq

Table III. Average plasmid-loss modeling results for biofilm cultures.

Set #	Number of runs at each growth rate	Average specific growth rate (h^{-1}) [Eq. (3)]	Average detachment rate (h^{-1}) [Eq. (4)]	Average plasmid-loss factor [Eq. (9)]	Average acetate concentration (m*M*)
1	3	0.22 ± 0.013	0.10 ± .03	0.029 ± 0.006	4.8
2	3	0.21 ± 0.026	0.17 ± .035	0.036 ± 0.007	8.2

(3)] and biofilm detachment parameters [Eq. (4)] and is shown below in its entirety.

$$p = \frac{\left[\begin{array}{c} \dfrac{dB^-}{dt} - \dfrac{(0.49\,S)}{\left(4.5 + S + \dfrac{S^2}{9.8}\right)} \\ + 2\left(1/a\,\dfrac{(X_{bi} - X_{bi-1})}{(t_i - t_{i-1})} + D/2(X_{bi} - X_{bi-1})\right)B^- \end{array}\right]}{\left[\dfrac{0.49\,S}{\left(4.5 + S + \dfrac{S^2}{9.8}\right)}\,B^+\right]} \quad (7)$$

Figure 5 illustrates that the population dynamics and distributions exhibited in the biofilm cultures are the same as that found in the detached effluent cells. This result is important, for it verifies that there is no preferential biofilm detachment of p(−) or p(+) cells in either of the studies. This verification helps support the validity of the plasmid loss model and the results reported here.

Comparison between the plasmid loss factors found for the previously reported suspended continuous cultures (Sharp et al., 1997) and the biofilm cultures of (17616-TOM_{31c}) are shown in Figure 7, plotted as a function of growth rate. The results indicate that there is little or no significant difference in the probability of plasmid-loss between the two culture types. Results suggest that there is no advantage or disadvantage to biofilm growth for this plasmid/host system. These results are contrary to Huang et al. (1993), who found that growth in biofilm culture created a higher segregational plasmid loss vs. suspended culture in a high copy number plasmid/host system. The higher copy number and marker gene system, inherent to the pMJR2770 plasmid, used by Huang et al., is highly unstable and its expression is physiologically detrimental to its host. Thus, the results seen in the Huang et al. culture are considered a consequence of the specific industrial plasmid/host system. Conversely, the 17616 (TOM_{31c}) is a plasmid/host system derived from an environmental isolate and involves only a transposon mutation and a natural transconjugation; hence, a major conflict between gene expression and biofilm formation was not anticipated.

In many industrial and pharmaceutical plasmid/host systems, plasmid instability is expected and compensated for by using highly controlled reactor systems. In bioremediation applications, a stable, highly active plasmid/host system is desired for both regulatory and process control purposes. Our results provide evidence for higher stability among biofilm cultures of plasmid/host systems derived from environmental isolates. In addition, the comparison between continuous culture and biofilm culture plasmid stability results are contrary to the conventional belief that net plasmid-loss decreases in biofilm cultures due to the occurrence of plasmid transfer and slower growth rates in the biofilm. One possible explanation is that the biofilm cells are losing their plasmids at a higher rate than the suspended cells, but an increase in plasmid transfer from plasmid-bearing to plasmid-free cells within the biofilm negates the higher loss rate resulting in an equivalent net plasmid-loss.

Results from other suspended and biofilm continuous culture experiments (Sharp, 1995) using other non-selective growth substrates (LBG and phthalate) also exhibited considerable plasmid loss, indicating that the plasmid loss noted here is not an artifact of acetate growth, but is either a consequence of continuous culture, which is suggested by the work of others (Dwilvedi et al., 1982; Primrose et al., 1984), or an inherent segregational loss process associated with this plasmid/host system.

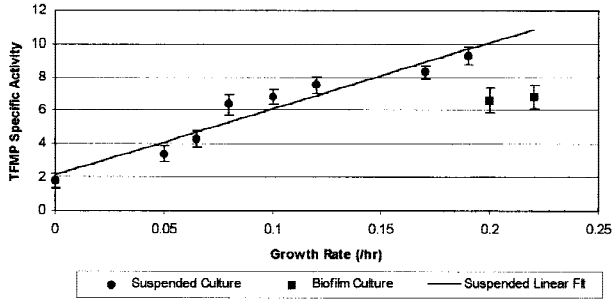

Figure 6. Comparison of true TOM specific activities for suspended and biofilm cultures of *P. cepacia* 17616-(TOM_{31c}).

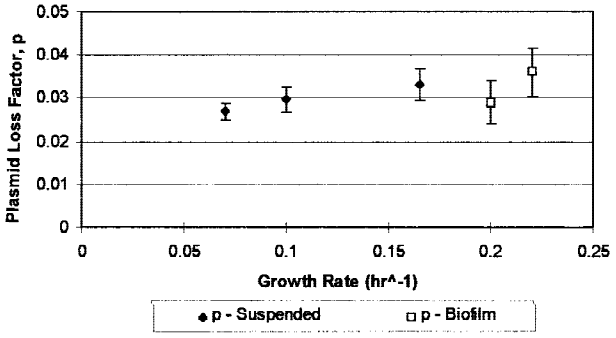

Figure 7. Comparison of plasmid loss factors for suspended and biofilm cultures of *P. cepacia* 17616-TOM_{31c}.

Physiological differences among attached cells and suspended cells could always play a role in the increase or decrease in plasmid retention within a given bacterial culture. With this specific plasmid/host system, and the environmental and growth conditions under which they were studied, it appears that the plasmid TOM_{31c} is equally stable in both suspended and biofilm cultures of the transconjugant 17616 (TOM_{31c}). The high rate of apparent plasmid-loss in both the biofilm and suspended cultures indicates that some form of selection for plasmid-bearing cells must be applied periodically if the culture is to maintain a dominant and active TCE degrading culture. Some possibilities for maintaining TOM_{31c} in the biofilm cultures include: periodic use of phenol as the primary growth substrate, dosing of Kanamycin to perform post-segregational killing of plasmid-free cells, and the incorporation of TOM_{31c} into a more stable host.

SUMMARY

Incorporation and expression of the *Tom* pathway in biofilm cultures of the transconjugant *Burkholderia cepacia* 17616 (TOM_{31c}) was demonstrated. Under selective growth conditions, Tom specific activity was maintained during long-term growth in biofilm cultures. The Tom specific activity of the 17616 (TOM_{31c}) biofilm cultures was found to be slightly less than that found in suspended cultures, indicating a slightly detrimental effect of attached growth.

Plasmid loss in 17616 (TOM_{31c}) in biofilm cultures was considerable, resulting in an order-of-magnitude decrease in plasmid-bearing cells over a 20–30 d period in both culture types. This degree of plasmid loss resulted in the critical loss of the TCE degradative phenotype, which would have a profound negative effect on the performance of a TCE-degrading bioremediation technology. No significant difference in the probability of plasmid loss between non-selective biofilm cultures and previously reported non-selective suspended cultures was found. Our studies show a definite need for plasmid selection and process control methods aimed at balancing plasmid loss and activity to produce an effective TCE degrading culture of 17616 (TOM_{31c}) for use in bioremediation technologies. These studies illustrate a need for further examination of plasmid loss in other environmentally relevant plasmid/host systems to determine the plasmid/host interactions that can affect the performance and efficiency of biodegradation technologies.

NOMENCLATURE

μ	specific growth rate ($time^{-1}$)
μ_{max}	maximum specific growth rate ($time^{-1}$)
K_s	Monod half-saturation constant (mMolar or mg/L)
K_i	Andrews substrate inhibition half-saturation constant (mMolar or mg/L)
[TCE]	concentration of TCE (mMolar or mg/L)
S	concentration of growth substrate (mMolar or mg/L)
X^+	plasmid-bearing cell concentration (cells/mL)
X^-	plasmid-free cell concentration (cells/mL)
t	time (min, h, or d)
p	plasmid loss factor
D	dilution rate (/time)
B^+	plasmid-bearing biofilm cell density ($cells/cm^2$)
B^-	plasmid-free biofilm cell density ($cells/cm^2$)
K_{det}	the detachment rate ($time^{-1}$)
X_{bi}	effluent bulk-fluid cell concentration at time point i (cells/mL)
X_{bOi}	influent bulk-fluid cell concentration at time point i (cells/mL)
a	specific area (surface area/volume of reactor)
dB-/dt	change in plasmid free cell number over time ($cells/cm^2$-d)
dB^+/dt	change in plasmid bearing cell number over time ($cells/cm^2$-d)

References

Alvarez-Cohen, L., McCarty, P. L. 1991a. Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene transformation by methanotrophic resting cells. Appl. Environ. Microbiol. **57**: 1031–1037.

Alvarez-Cohen, L., McCarty, P. L. 1991b. Effects of toxicity, aeration, and reductant supply on trichlorethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. **57**: 228–235.

Bailey, J. E., Ollis, D. E. 1986. Biochemical engineering fundamentals, 2nd edition. McGraw-Hill, New York.

Camper, A., Jones, W. 1997. Estimated biofilm organism growth rates as influenced by distribution system parameters. Biofouling, in press.

Characklis, W. G., Marshall, K. C. (eds.) 1990. Biofilms. Wiley, New York.

Cheng, H-P., Lessie, T. G. 1994. Multiple replicons constituting the genome of *Pseudomonas cepacia* 17616. J. Bacteriol. **176**: 4034–4042.

Dwilvedi, C. P., Imanaka, T., Aiba, S. 1982. Instability of plasmid harboring strain *E. coli* in continuous culture. Biotechnol. Bioeng. **24**: 1465–1468.

Dykhuizen, D. E., Hartl, D. L. 1983. Selection in chemostats. Microbiol. Rev. **47**: 150–168.

Ensley, B. D. 1986. Stability of recombinant plasmids in industrial microorganisms. CRC Critical Rev. Biotechnol. **4**: 263–277.

Fenell, D. E., Nelson, Y. M., Underhill, S. E., White, T. E., Jewell, W. J. 1993. TCE degradation in methanotrophic attached-film bioreactor. Biotech. Bioeng. **42**: 859–872.

Finette B. A., Subramanian, V., Gibson, D. T. 1984. Isolation and characterization of *Pseudomonas putida* PpF1 mutants defective in the toluene dioxygenase enzyme system. J. Bacteriol. **160**: 1003–1009.

Folsom, B. R., Chapman, P. J. 1991. Performance characterization of a model bioreactor for the biodegradation of trichloroethylene by *Pseudomonas cepacia* G4. Appl. Environ. Microbiol. **57**: 1602–1608.

Folsom, B. R., Chapman, P. J., Pritchard, P. H. 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: Kinetics and interactions between substrates. Appl. Environ. Microbiol. **56**: 1279–1285.

Freedman, D. L., Gossett, J. M. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl. Enviro. Microbiol. **55**: 2144–2151.

Gibson, D. T., Yen, W. K., Liu, T. N., Subramanian, V. 1982. Toluene dioxygenase: A multi-component enzyme system from *Pseudomonas putida*, pp. 51–61. In: M. Nozaki, S. Yamamoto, Y. Ishimura, M. J. Coon, L. Ernster, R. W. Estabrook (eds.), Oxygenase and oxygen metabolism. Academic Press, New York.

Grandi, G., Mottes, M., Sagaramella, V. 1981. Specific pattern of instability of *E. coli* HisG gene cloned *Bacillus subtilis* via the *Staphylcoccus aureus* plasmid pCS194. Plasmid **6**: 99–111.

Huang, C. T., Peretti, S. W., Bryers, J. D. 1993. Plasmid retention and gene expression in suspended and biofilm cultures of recombinant *E. coli* DH5 (pMJR1750). Biotechnol. Bioeng. **41**: 211–220.

Huang, C. T., Peretti, S. W., Bryers, J. D. 1994. Effects of carbon-nitrogen ratio on biofilm formation and plasmid retention. Biotechnol. Bioeng. **44**: 329–336.

Inloes, D. S., Smith, W. J., Taylor, D. P., Cohen, S. N., Micheals, A. S., Robinson, C. R. 1983. Hollow fiber membrane bioreactors using immobilized *E. coli* for protein synthesis. Biotechnol. Bioeng. **25**: 2653–2681.

Jones, S. A., Melling, J. 1984. Persistence of pBR322-related plasmids in *E. coli* grown in chemostat cultures. FEMS Microbiol. Lett. **22**: 239–243.

Jones, I. M., Primrose, S. B., Ellwood, D. C. 1980. Maintenance of some ColE1-type plasmid in continuous culture. Mol. Gen. Genet. **180**: 579–584.

Kadam, K. L., Wollweber, K. I., Grosch, J. C., Jao, Y. C. 1987. Investigation of plasmid stability in amylase-producing *B. subtilis* using continuous culture. Biotechnol. Bioeng. **29**: 859–872.

Kaphammer, B., Kukor, J. J., Olsen, R. H. 1990. Cloning and characterization of a novel toluene degradative pathway from *Pseudomonas picketti* PKO1, abstr. K-145, p. 243. Abstr. 90th Annual Meeting Am. Soc. Microbiol. American Society for Microbiology, Washington, D.C.

Kumar, P. K., Maschke, H. E., Friechs, K., Schugerl, K. 1991. Strategies for improving plasmid stability in genetically modified bacteria in bioreactors. Trends Biotechnol. **9**: 279–284.

Kumar, P. K., Schugerl, K. 1990. Immobilization of genetically engineered cells: A new strategy for higher plasmid stability. J. Biotechnol. **14**: 255–272.

Lauffenburger, D. A. 1987. Bacteriocin production as a method of maintaining plasmid-bearing cells in continuous culture. Trends Biotechnol. **5**: 87–97.

Levin, B. R., Stewart, F. M., Rice, V. A. 1979. The kinetics of conjugative plasmid transmission: Fit of a simple mass action model. Plasmid **2**: 247–260.

Murgel, G. A., Lion, L. W., Acheson, C., Shuler, M. L., Emerson, D., Ghiorse, W. C. 1991. Experimental apparatus for selection of adherent microorganisms under stringent growth conditions. Appl. Environ. Microbiol. **57**: 1987–1996.

Nasri, M., Sayadi, S., Barbotin, J. N., Dhuster, P., Thomas, D. 1987. Influence of immobilization on the stability of pTG201 recombinant plasmid in some strains of *E. coli.* Appl. Environ. Microbiol. **53**: 740–744.

Noack, D., Roth, M., Geuther, R., Muller, G., Undisz, K., Hoffmeier, C., Gaspar, S. 1982. Maintenance and genetic stability of vector plasmids pBR322 and pBR325 in *E. coli* K12 strains grown in a chemostat. Mol. Gen. Genet. **184**: 121–124.

Nelson, M. J. K., Montgomery S. O., O'Neill, E. J., Pritchard, P. H. 1986. Aerobic metabolism of TCE by a bacterial isolate. Appl. Environ. Micro. **52**: 383–384.

Nelson, M. J. K., Montgomery, S. O., Mahaffey, W. R., Pritchard, P. H. 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Micro. **53**: 949–954.

Peretti, S. W., Bailey, J. E. 1987. Simulations of host-plasmid interactions in *E. coli:* Copy number, promoter strength, and ribosome binding site strength effects on metabolic activity and plasmid gene expression. Biotechnol. Bioeng. **29**: 316–325.

Primrose, S. B., Derbyshire, P., Jones, I. M., Robinson, A. 1984. The application of continuous culture to the study of plasmid stability. In: A. C. R. Dean, D. C. Ellwood, and C. G. T. Evans (eds.), Continuous Culture 8. Society of Chemical Industry/Ellis Horwood Limited, West Sussex, England.

Roth, M., Muller, G., Noack, D. 1980. Loss of the multi-copy resistance plasmid pBR325 from *E. coli* GY2354 pBR325 during continuous cultivation, pp. 143–146. In: S. Mitsuhasi, L. Rosival, and V. Krecmery (eds.), Antibiotic resistance: Transposition and other mechanisms. Springer-Verlag, New York.

Roth, M., Noack, D. 1982. Genetic stability of differentiated functions in *Streptomyces hygroscopicus* in relation to conditions of continuous culture. J. Gen. Microbiol. **128**: 107–114.

Sayadi, S., Nasri, M., Barbotin, J. N., Thomas, D. 1989. Effect of environmental growth conditions on plasmid stability, plasmid copy number, and C230 activity in free and immobilized *E. coli* cells. Biotechnol. Bioeng. **33**: 801–808.

Saye, D. J., Ogunseiten, O., Sayler, G. S., Miller, R. V. 1987. Potential for transduction of plasmids in natural freshwater environment: Effect of plasmid donor concentration and a natural microbial community on transduction of *Ps. aeruginosa*. Appl. Environ. Microbiol. **53**: 987–995.

Seo, J. H., Bailey, J. E. 1985. Effects of recombinant plasmid content on growth properties and cloned gene product formation in *E. coli*. Biotechnol. Bioeng. **27**: 1668–1674.

Sharp, R. R., Bryers, J. D., Jones, W. L., Shields, M. S. 1997. Activity and stability of a recombinant plasmid-borne TCE degradative pathway in suspended cultures. Biotechnol. Bioeng., In Press.

Sharp, R. R. 1995. Stability and expression of a plasmid-borne TCE degradative pathway in suspended and biofilm cultures. Ph.D. thesis, Montana State University.

Sherrat, D. J. 1982. Some stochastic models for plasmid copy number. Theor. Pop. Biol. **23**: 241–256.

Shields, M. S., Moody, T., Gerber, R., Sharp, R. S. 1993. Unpublished data from field study.

Shields, M. S., Reagin, M. J., Gerger, R., Campbell, R., Sommerville, C. 1995. TOM, a new aromatic degradative plasmid from *Burkholderia* (*Pseudomonas*) *cepacia* G4. Appl. Environ. Microbiol. **61**: 1352–1356.

Shields, M. S., Montgomery, S. O., Chapman, P. J., Cuskey, S. M., Pritchard, P. H. 1989. Novel pathway of toluene catabolism in the TCE degrading bacterium G4. Appl. Environ. Microbiol. **55**: 1624–1629.

Shields, M. S., Montgomery, S. O., Cuskey, S. M., Chapman, P. J., Cuskey, S. M., Pritchard, P. H. 1991. Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. Appl. Environ. Microbiol. **57**: 1935–1941.

Shields, M. S., Reagin, M. J. 1992. Selection of a *Pseudomonas cepacia* strain constitutive for degradation of trichloroethylene. Appl. Environ. Microbiol. **58**: 3977–3983.

Speital, G. E., Leonard, J. M. 1992. A sequencing biofilm reactor for the treatment of chlorinated solvents using methanotrophs. Wat. Environ. Res. **64**: 712–719.

Srinivasan, R., Stewart, P., Griebe, T., Chen, C. I., Xu, X. M. 1995. Biofilm parameters influencing biocide efficacy. Biotechnol. Bioeng. **46**: 553–560.

Stewart, G. J., Carlson, C. A. 1986. The biology of natural transformations. Ann. Rev. Microbiol. **40**: 211–235.

Strandberg, G., Donaldson, T. L., Farr, L. L. 1989. Degradation of trichloroethylene and *trans*-1,2-dichloroethylene by a methanotrophic consortium in a fixed-film, packed-bed bioreactor. Environ. Sci. Technol. **20**: 167–174.

Summers, D. K. 1991. The kinetics of plasmid loss. Trends Biotechnol. **9**: 273–278.

Taxis du Poët. P., Arcand, Y., Bernier, R., Barbotin, J.-N., Thomas, D. 1987. Plasmid stability in immobilized and free recombinant *E. coli* JM 105(pKK223-200): Importance of oxygen diffusion, growth rate, and plasmid copy number. Appl. Environ. Microbiol. **53**: 1548–1555.

Tiedje, J. M., Colwell, R. K., Grossman, Y. L., Hodson, R. E., Lenski, R. E., Mack, R. N., Regal, P. J. 1989. The planned introduction of GEOs: Ecological considerations and recommendations. Ecology **70**: 298–315.

Tsien, H. C., Brusseau, G. A., Hanson, R. S., Wackett, L. P. 1989. Biodegradation of TCE by *Methylosinus trichosporium.* Appl. Environ. Microbiol. **55**: 3155–3161.

Uhlin, B. E., Nordstrom, K. 1978. A runaway-replication mutant of plasmid R1drd-19: Temperature-dependent loss of copy number control. Mol. Gen. Genet. **165**: 167–179.

Vandenbergh, P. A., Kunka, B. S. 1988. Metabolism of volatile chlorinated aliphatic hydrocarbons by *Pseudomonas florescens.* Appl. Environ. Microbiol. **54**: 2578–2579.

Vogel, T. M., McCarty, P. 1985. Biotransformation of tetrachloroethylene to TCE, DCE, VC, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. **49**: 1080–1083.

Wackett, L. P., Gibson, D. T. 1988. Degradation of TCE by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54**: 1703–1708.

Wood, T. K., Kuhn, R. H., Peretti, S. W. 1990. Enhanced plasmid stability through post-segregational killing plasmid-free cells. Biotechnol. Tech. **4**: 39–44.