Survival and Activity of a 3-Chlorobenzoate-Catabolic Genotype in a Natural System

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A chlorobenzoate-degrading Alcaligenes strain, BR60, was introduced to flowthrough lake microcosms and exposed to 3-chlorobenzoate (3Ca) concentrations from 0 to 25 μM. A DNA probe specific for BR60 chlorobenzoate catabolic genes was used with the most probable number (MPN) technique to enumerate bacteria harboring this genetic information. This MPN-DNA hybridization method combined with [U-14C]3Ca uptake rate measurements allowed the correlation of the size and activity of a specific catabolic population in a natural mixed community for the first time. An experiment involving the release of a streptomycin-resistant strain of BR60 indicated that estimates of bacteria carrying the introduced catabolic genotype often outnumbered plate count estimates of viable BR60 by as much as 3 orders of magnitude, particularly when 3Ca inputs were high. The MPN-DNA hybridization method provided catabolic population estimates highly correlated to 3Ca exposure levels and the [U-14C]3Ca uptake rates in the microcosms. Plate counts of BR60 were poorly correlated with both 3Ca exposure levels and uptake rates. In the absence of chlorobenzoate selection, the catabolic genotype declined to very low levels by the MPN-DNA hybridization technique after 8 weeks in the microcosms.

Isolated bacterial populations form the basis of our knowledge of the metabolism, genetics, and ecology of chlorobenzoic aromatic degraders (17). The population dynamics and natural activity of these organisms within complex microbial communities remain poorly understood, primarily because of technical difficulties of making measurements in situ. In environments such as the water and benthos of rivers or lakes, chlorinated organic contaminants may occur at or below micromolar levels. Although biodegradation of synthetic organic contaminants does occur at low concentrations (11), studies suggest that the organisms responsible differ from those operating at higher concentrations (18). The usefulness of laboratory-selected strains as inocula in natural situations has been demonstrated in only a handful of cases (2, 3, 9).

Natural conditions may present a number of problems for laboratory-selected contaminant degraders reintroduced into the environment. Low substrate concentrations may not support the growth of these specialized strains, or the introduced organisms may abandon metabolism of the contaminant in favor of other substrates (6). Naturally occurring toxins, predators, and interspecific competition for nutrients may all reduce the survival of the introduced strain (10, 12, 19). On the other hand, alternate carbon sources available in natural ecosystems may enhance the survival of introduced strains and hence the degradation of low-level contaminants (21). All of these scenarios are based on the characteristics of the introduced strain. However, the genetic information for catabolism of many contaminants is frequently carried on plasmids or other mobile genetic elements with a potential for transfer to other bacteria. Interspecific transfer of catabolic genes has been documented in simulated natural conditions in continuous culture (20). The transfer of genetic information to new hosts meant that the survival characteristics of the original host may not totally determine the fate of an introduced catabolic genotype. This study involves a naturally occurring 3-chlorobenzoate (3Ca) degrader, Alcaligenes sp. strain BR60. The strain carries a plasmid, pBR60, that encodes 3Ca degradation (24). We released BR60 into flowthrough freshwater and sediment microcosms designed to closely simulate the conditions in a shallow bay. The microcosms were dosed with different amounts of 3Ca. The frequency of the catabolic genotype in each microcosm was monitored by using DNA probes specific for the catabolic region of pBR60. Because of the inherent mobility of this genetic material, the data describe the survival and activity of a specific genotype, not specific bacteria, in a natural system. We present evidence here that the frequency of the catabolic genotype differs from that of the originally introduced bacteria and that the genotype frequency is of greater importance to 3Ca metabolism in the system than the survival of the original inoculum.

MATERIALS AND METHODS

Source of the inoculum. Alcaligenes sp. strain BR60 was isolated from sediment of Bloody Run Creek, a chlorobenzoate- and chlorophenol-contaminated creek draining the Hyde Park industrial landfill site in Niagara Falls, N.Y. Details on the isolation and genetic makeup of this organism have been published (24). BR60 was isolated as a chlorobenzoate-resistant species isolated on 3Ca as the sole carbon and energy source that harbored an unstable 85-kilobase-pair plasmid, pBR60, carrying the catabolic genotype. A mobile 14-kilobase-pair region of pBR60 (the ubp region) harbors genes central to chlorobenzoate metabolism.

Microcosm design. Queen’s University Biological Station is situated on Lake Opinicon, a marble-granite-based lake found on the Frontenac Axis of eastern Ontario. The toxicology facility at this station was used to set up 12 microcosms representative of an epilimnetic sediment-water interface. The microcosms were large aquaria (72 by 40 by 22 cm [height]) containing superficial sediments, complete with small macrophytes, detritus, and smalls, taken directly from a shallow bay of the lake. A 5-cm layer of epilimnetic sediment was allowed to settle and was not subsequently disturbed.
The 50 liters of overlying water was aerated gently with porous stones. Epiplastic lake water was continuously pumped from an intake pipe in the middle of an adjacent 2-m-deep bay into an overflowing header tank before delivery to the various microcosms at a controlled dilution rate. 3-Cba sterile aqueous solutions were also delivered at fixed rates to selected tanks. Overflow water from experimental tanks was passed through a carbon filter before it entered a seepage bed. No nutrients were added, and all experiments took place at ambient temperatures.

Initial release experiments. In July and August of 1987, microcosms were set up and dosed with 3Cba at four different approximate concentrations. Lake water was piped from the header tank to the microcosms by using aquarium air tubing constricted with plastic clamps such that the dilution rate approximated 0.05h. 3Cba was delivered from sterile 4-mL reservoirs by commercially available intravenous apparatus. Four microcosms received 3Cba at 0.04 mL/min, and four received 3Cba at 0.1 mL/min. Two microcosms were dosed by flowing gauze bags of powdered 3Cba in the water. Two microcosms received no 3Cba. These treatments exposed microcosms to mean 3Cba concentrations ranging from 0 to 36 µM with a high level of variation due to invertebrate water flow rates.

Alcaligenes sp. strain BR60 was introduced to one-half of the tanks (representative of the full range of 3Cba concentrations) on 29 June 1987. Cultures in 100 mL of medium A (23) and 3 mL 3Cba plus approximately 20 g of white, sterilized sand were grown to stationary stage and then used to inoculate the microcosms. All microcosms were monitored for 8 weeks.

Viable counts were made by using 2 mL 3Cba-0.005% yeast extract and 2 mL 3Cba-0.005% yeast extract agar plates. Water or sediment samples were diluted in medium A. The population of bacteria carrying the catalytic genotype in each microcosm was determined by using DNA probes (described below) every 2 to 3 weeks. Two determinations were made each month, one using a general medium (10-15% yeast extract) in the initial culture step and the other with a more selective medium (2 mL 3Cba plus 0.005% yeast extract). 3Cba uptake rates in microcosm waters were determined in mid-August and again at the close of the experiment (September, data not shown below). Total phosphorus, nitrate, and dissolved organic carbon levels in the intake water were determined biweekly by the Ontario Ministry of the Environment. Dose-response quantification experiments. Technical improvements in the use of chlorobenzene delivery systems were made in 1986 to allow finer control on the feed concentration of 3Cba. Water flow rates from the header tank were controlled by passage through fine-gauge stainless steel needles. To eliminate problems of clogging, lake water was initially passed through 170-µm mesh screen. Dilution rates were set at 0.01h by adjusting the flow rate of the gauze needle and a water height of 3 cm above the needle tip. 3Cba was delivered from sterile reservoirs to the water delivery lines via a 10-cm 10-channel peristaltic pump (model 501U; Watson-Marlow) at the rate of 0.008 mL/min. Reservoir concentrations were adjusted to give 3Cba feed concentrations ranging from 10 µM to 25 µM. Aquaria were sealed with pleathose lids containing input and output tubes sealed in place. These lids were designed to eliminate the transfer of air or water spray between microcosms.

A naturally occurring streptomycin-resistant strain of Alcaligenes sp. strain BR60 was used for microcosm inoculation. The strain was grown to stationary phase on medium A supplemented with 2 mL 3Cba-15 g of streptomycin per mL and introduced at 100,000 cells per mL to all microcosms on 25 June 1988. Incorporation cell densities were determined by plating on 3Cba-streptomycin and 3Cba agar. Water and sediment viable counts were made weekly on agar containing 3 mL 3Cba as the sole carbon source and on agar similar containing 25 µg of streptomycin per mL. The populations carrying the catalytic genotype were determined weekly as described below by using only a general growth medium (0.1% yeast extract, 0.1% tryptone). 3Cba uptake rates in the microcosms were determined at the close of the experiment 4 weeks after inoculation.

Detection of the catalytic genotype. The presence of BR60 and/or bacteria carrying DNA homologous to the yb869 catalytic dbp region was detected by using radiolabeled fragments of that region. To quantify organisms hybridizing with our probe, a most probable number (MPN-DNA hybridization method adapted from Frederickson et al. (25) was used. Undiluted microcosm water or diluted sediment (10-3) was inoculated with five replicates for each sample and diluted a further 3 or 4 orders of magnitude in microdilution plates containing a growth medium. After 4 to 5 days of growth, contents of the plates were filtered onto nitrocellulose or nylon (Hybond; Amersham Corp.) membranes by using a vacuum manifold designed for the filtration of microdilution plate wells (Blasicher & Schnett, Inc.). DNA from the enriched populations in the wells was fixed in the membrane in situ by exposure to 10% sodium dodecyl sulfate, denaturing solution (0.5 M NaOH, 1.5 M NaCl), neutralizing solution (0.5 M Tris, 1.5 M NaCl), and SSPE (0.18 M NaCl-0.1 M NaHPO4-0.001 M EDTA [pH 7.3]), followed by drying and 2 h of baking at 80°C for the nitrocellulose membranes, or 5 min of exposure to short-wave UV light for nylon membranes (13). Membranes were stored dry in sealed packages at -4°C until hybridization. Cleared fragments of the dbp region were used to make DNA probes. A plCUB library of EcoRI and HindIII pBR60 fragments (27) was used as probe. Labeled EcoRI fragments 11 or 5 of HindIII fragment 2 were used as DNA probes for the dbp region. plCUB plasmids were isolated from E. coli JM101 by using alkaline lysis and were purified by chromatography on Sepharose 4B columns (Pharmacia). DNA was precipitated from the plasmid solution by cutting out using either EcoRI or HindIII and loaded onto a low-temperature-melting-point agarose gel (Seaplaque agarose; FMC Corp., Marine Colloids Div, for electrophoresis. The pBR60-derived fragment was cut from the agarose, diluted 1:3 with sterile distilled water, and dried until no further water was seen needed. The probe was prepared by denaturing the fragment at 100°C for 5 to 10 min and labeling with [32P]dCTP (Amersham) by the random primer-Klenow polyadenylation method (4). The labeled probe was separated from unincorporated [32P]dCTP on a Sephadex G-50 column equilibrated in 10 mL of 0.1 M Tris-HCl (pH 7.5) and then applied to a 10 cm x 2 cm column of Bio-Gel P-4 (Bio-Rad). The column was washed with 10 mL of 0.1 M Tris-HCl (pH 7.5) and then eluted with 10 mL of 0.1 M Tris-HCl (pH 8.0) and 10 mL of 0.1 M Tris-HCl (pH 8.0). The column was then washed with 10 mL of 0.1 M NaCl (pH 7.5) and 0.02 M sodium citrate (105 cm 20 ml of hybridization solution. Hybridizations were carried out using stringent conditions at 30°C formamide at 42°C as described previously (24). Three washes were carried out using 0.1% sodium dodecyl sulfate and 0.1x SSC (10 ml 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, followed by one wash at 55°C. After exposure to Kodak X-Omat AR X-ray film, wells showing hybridization of a density comparable to that of BR60 control wells were counted, and an estimate of probe-positive bacteria was calculated from MPN tables (25).

Uptake rates of chlorobenzene. The heterotrophic poten- tial of the microcosm waters was determined by using
3C-labeled 3Ca (specific activity, 12.3 mCi/mmol; Sigma Radiochemicals). Microcosm water was pipetted into ribber-stoppered glass test tubes in 3-mL replicate samples for each of five concentrations of added 3C-labeled 3Ca, ranging from 1 to 100 μM. Tubes were incubated at room temperature for a fixed period (1 to 3 h) before the contents were filtered (0.22-μm-pore-size membranes; Nucleopore Corp.) by using a vacuum manifold. The filters were rinsed with 6 mL of distilled water, dissolved in 0.5 mL of ethanolamine, and counted in an LKB scintillation counter by using a 15% methanol-0.4% PPO-BisMIB (premixed; ICN Radiochemicals)-toluene scintillation cocktail (25). Uptake controls were carried out by using formaldehyde-treated water. Maximum uptake rates were estimated by assuming that uptake followed Michaelis-Menten kinetics such that $V = \frac{V_{\text{max}} \times S}{K_s + V_{\text{max}}}$, where $S$ is the substrate concentration, $V$ is the uptake rate, $V_{\text{max}}$ is the maximum uptake rate for the system, and $K_s$ is the half-saturation concentration. $V_{\text{max}}$ was derived from the inverse of the slope of the curve plotted.

Chlorobenzene concentrations. The concentrations of 3Ca in reservoirs used to deliver the chemical to the microcosms and the initial concentrations of 3Ca in the waters of the microcosms were determined by high-pressure liquid chromatography. We used a Waters 501 solvent pump with a Nova-Pak C18 reverse-phase column combined with a Lambda-Max model 481 LC spectrophotometer set at 220 nm to detect 3Ca in aqueous samples. The solvent used was a 1:1 mixture of methanol and 1% acetic acid.

RESULTS

Microcosm parameters. Background levels of organisms hybridizing with our DNA probes were negligible in Lake Opinicon waters and sediments before the introduction of BR60. Lake sediment samples obtained from six locations around the lake in March 1987 were plated on 2 mM 3Ca-0.05% yeast extract agar. Colonies forming on this medium were hybridized in situ on nitrocellulose filters to a whole plasmid pBR32 DNA probe. Omitting non-specific binding was detected for some of the colonies. In June 1987 lake water and sediment were diluted in general medium MPN microdilution plates, and subsequent growth was probed with EcolI fragment 11. Again, omitting hybridization, indicative of non-specific binding, was detected.

Lake water bioassay input rates for July and August 1987 varied from 2 to 62 μg/L and averaged 30 ± 20 (standard error) μg/L. Dissolved organic carbon and total Kjeldahl nitrogen levels were measured constant at 5.2 ± 0.7 and 0.52 ± 0.09 mg/L, respectively. Changes in these nutrient parameters had no discernible effect on BR60 populations. Ambient temperatures varied between 20 and 32°C over both the 1987 and 1986 experimental periods.

Chlorobenzene dosages. Results are presented and discussed with reference to the chlorobenzene dosage received by the various microcosms. This is always reported as the concentration of chlorobenzene in the incoming lake water, termed the 3Ca feed rate. In 1987 the incoming water and 3Ca did not mix before delivery to the tanks, and in 1988 they did, but in both cases the 3Ca feed rate was calculated as 3Ca reservoir concentration × 3Ca input rate/water input rate for those microcosms receiving 3Ca from the reservoirs of aqueous solutions.

Initial-release experiments—1987. For the tanks with 3Ca delivered as a solid suspension, the 3Ca concentration was measured in the microcosms after equilibration with the substrate and before introduction of BR60. There was a significant amount of variation over a 24-h period in the water input rate, and it was adjusted daily to compensate for this problem. In addition, there was some variation over a much longer period of time in the 3Ca input rates. Both these sources of variation made the estimated 3Ca feed rate fairly approximate, and no attempt was made to quantify results with respect to this parameter.

(a) Growth of BR60 on agar plates. On 29 June 1987, BR60 was introduced into the microcosms at approximately 10^6 CFU/mL. On 3 July 1987 BR60 colonies were discernible on the 3Ca-0.05% yeast extract plates from the inoculated microcosms. Eight days after introduction, similar colonies were discernible on plates from uninoculated control microcosms, and thereafter MPN probe-positive counts in the control microcosms rose to levels not significantly different from those in the inoculated microcosms. Intermicrocosm transfer of bacteria via aerosols was suspected, as all apparatus were uncovered. Thereafter the uninoculated tanks were treated as duplicates. The size of the original inoculum of BR60 did not seem to be an important factor in establishing BR60 in the microcosms.

(b) Detection using MPN-DNA hybridization. The average population levels of organisms carrying DNA homologous to our probe (termed probe positive for brevity) in the microcosms are depicted in Fig. 1. Each bar shows the average of the log probe-positive population from four estimates taken over the 2-month period. Estimates depended on the medium used in the initial enrichment step in the MPN-microdilution plates. The levels estimated on general media were higher than those estimated on selective media. Estimates derived from general media tended to remain the same (for [3Ca] < 20 μM) or to increase (for [3Ca] > 23 μM) over the 2-month sampling period. In contrast, estimates derived from selective media increased for 3Ca exposures of 56, 31, and 17 μM but remained the same or decreased in all other microcosms.

![Figure 1: Average log probe-positive organisms for 1987 initial release experiment versus average 3Ca feed concentrations in microcosms. Error bars are standard errors.](image-url)
By this technique, the theoretical lowest detectable level of probe-positive organisms was 8 cells per ml in the waters or 60 cells per ml in the sediments. Probe-positive organisms persisted in water and sediments of all the microcosms except those lacking 3Cba inputs for the full 56 days of the experiment. In both the inoculated and uninoculated microcosms without 3Cba, probe-positive counts in the sediments dropped to undetectable levels after 41 days. In the overlying waters, probe-positive organisms persisted at less than 100 cells per ml after 56 days. Their persistence in the waters may have been due to continuing aerosol contamination from adjacent high-3Cba-dosed tanks.

After release of Alcaligenes sp. strain BR60, average probe-positive estimates over the 8-week period were clearly related to the concentration of chlorobenzene delivered to the microcosms (Fig. 1). The variance in probe-positive counts for each microcosm was proportional to the mean, so a log transformation was performed before any analyses of these data. After transformation, there was a significant difference in the MPN probe-positive counts between microcosms in both the water and sediments (analysis of variance: $F_{(1,29)} = 3.89, P < 0.05; F_{(1,24)} = 3.56, P < 0.05$, respectively). The mean log probe-positive estimates were highly correlated to the log of the estimated 3Cba feed concentration, and those obtained from the use of general media were more highly correlated than those from selective media (general media: $r = 0.85$; selective media: $r = 0.72$; sediments: $r = 0.49$; sediments $r = 0.45$). Regression parameters were not calculated for these relationships because of the large amount of error associated with estimating the 3Cba concentration. In addition, in the higher-dosage microcosms, the MPN probe-positive counts were minimum estimates because dilutions were not sufficient to yield a definite estimate from the MPN table. The variances of the MPN estimates were also indicative of an association between these variables but should not be used to estimate the variance explained in the probe-positive estimates by the 3Cba feed rate. This was estimated in the 1988 experiment.

(vi) Observed colonization rates. The heterothrophic potential of the water in the microcosms was determined between 10 August and 2 September 1987. Two measurements were performed on each tank at temperatures between 17 and 20°C. The estimate of $V_{max}$ for 3Cba uptake reflected the estimated maximum 3Cba feed concentration ($p = 0.85$). In addition, $log(V_{max})$ was highly correlated to the log (probe-positive organisms) estimate—again, more so to the general media estimate than to the selective media estimate ($r = 0.79$ versus $r = 0.48$). These results are discussed further in relation to the colonization experiment.

Due-response questionnaires-1988. Technical improvements made to the microcosm fluid input systems stabilized the flow rates dramatically and allowed much more precise control of the 3Cba feed concentrations. Input water flow rates did not vary significantly over the 4-week period, remaining at 7.25 ± 0.67 ml/min. 3Cba microcosm concentrations were measured after 4 weeks by high-performance liquid chromatography and showed no change from their initial concentrations.

(vi) Viable plate counts of BR60. After inoculation into the microcosms, 3Cba-streptomycin agar was evenly dispersed in the waters and sediments 4 days later. It formed distinct colonies on 3Cba or 3Cba-streptomycin agar, having translucent cream-colored colonies with slightly irregular margins and exhibiting the smooth-irregular colony morphology seen in the laboratory (25). On 3Cba-streptomycin agar, BR60-like colonies were virtually the only growth seen. On 3Cba agar, many colonies formed, but BR60-like colonies were evident from their much larger size and rapidity of formation. BR60-like colonies recovered from some microcosms exhibited a variance in chlorobenzene metabolism evident from the accumulation of dark-brown chlorocatechol oxidation products in the center of the colony. Subsamples of these colonies and culturing in 3Cba liquid culture resulted in normal non-chlorobenzene-accumulating colonies. BR60 grew more quickly after sampling from the microcosm than after repeated culturing in the laboratory in medium A plus 3Cba. According to viable counts made on 3Cba streptomycin agar 3 days after inoculation, BR60 populations averaged 1.0 ± 10^6 CFU/ml in microcosm waters and 1.1 ± 10^6 CFU/ml in the superficial sediments. The highest populations were found in microcosms receiving 10 nM, 40 nM, 700 nM, 10 Î¼M, and 20 Î¼M 3Cba, exhibiting no dependence on chlorobenzene concentration. After 14 days, counts across all tanks averaged 68 CFU/ml in the waters and 4.0 ± 10^6 CFU/ml in the sediments. No colony-forming BR60 were detected in microcosm waters after 32 days, and sediment populations had dropped to 1.7 ± 10^5 CFU/ml. There was no statistical difference between microcosms with respect to viable BR60 counts according to an analysis of variance ($F_{(10,94)} = 0.54, 66, n$sediments, $F_{(10,94)} = 0.60, n$sediments, $F_{(10,94)} = 1.14, n$sediments, indicating that a loss of streptomycin resistance was not the cause of this result.

(ii) Detection using MPN-DNA hybridizations. Probe-positive estimates were determined by using a general medium. The counts obtained gave considerably higher estimates of density. Note that these are the viable counts of BR60 in some microcosms. The MPN technique can yield different estimates of a cultivable population than plate counts do. In our experience with BR60 growing in pure culture, MPN population estimates never diverge more than a factor of 3. In contrast, the MPN probe-positive estimates from the microcosms were often orders of magnitude higher than the viable counts of BR60 (Table 1). The actual discrepancy in values is depicted in Fig. 2. The overestimation was most obvious in the waters in which viable BR60 were below detectable levels, and no probe-positive counts were significant. This phenomenon was not a function of the sample volume used in the two methods, since a triplicate viable count took 150 μl of sample and an MPN probe-positive estimate took 100 μl. Probe-positive populations were significantly different be-

<table>
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<tr>
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</tr>
<tr>
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<td>56</td>
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<td>T3</td>
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* The ratio is equal to infinity (+) in some cases (B90, count, 0).
Fig. 2. MPN-DNA probe-positive (†) versus counts minus viable BR66 counts plotted against 3ChA feed concentration for 1988 microcosms. Symbols: O, sediments; O, water.

Fig. 4. 3ChA uptake rate (Vmax) in microcosm waters versus log[3ChA (feed concentrations)]. The x axis is shown as log scale for clarity. Linear relationship is r² = 0.92; Vmax = 1.48[3ChA] + 3.99.

between microcosms in both the water (F(10,25) = 2.41, P = 0.05) and the sediments (F(10,22) = 7.11, P < 0.001) according to an analysis of variance of the log-transformed data. The mean log estimates show a significant regression on the log of the 3ChA feed concentration (Fig. 3). Untransformed estimates were regressed on 3ChA feed concentration in order to determine the average yield in terms of probe-positive cells per mg of 3ChA. They averaged 2.35 × 10⁶ cells per mg in the microcosms waters and 1.07 × 10⁶ cells per mg in the sediments. In continuous culture of BR66 in sterile creek water containing 200 μM 3ChA, the yield averaged 4.7 × 10⁶ cells per mg (25).

(b) Chlorobenzene uptake rates. 3ChA uptake rates in the microcosm waters were measured on 24 July at the end of the 4-week experiment. Vmax was significantly correlated to the 3ChA feed concentration as expected (Fig. 4; r² = 0.92, Vmax = 1.88 [3ChA] + 3.99). Vmax did not increase above background levels until the 3ChA concentration was greater than 1 μM. Log Vmax was again significantly correlated to the log of the probe-positive counts (Fig. 5). The relationship between probe-positive organisms and Vmax is similar for the 1987 initial release experiments and for the 1988 experiment, but the slopes of the regressions are significantly different (F = 12.4, P = 0.001). In 1988, Vmax was lower for a given population of probe-positive organisms than in 1987.

The 3ChA uptake rates could not be predicted from the plate counts of BR66 in the waters. Correlations between Vmax and microcosm average plate counts are weak (r² = 0.47 and 0.25 for counts on 3ChA and 3ChA-streptomycin agar, respectively). During the week that Vmax was actually measured, viable counts had dropped to 0 in most of the microcosms, including the one receiving 10 μM 3ChA. For the four microcosms retaining viable BR66, no relationships to either 3ChA exposure or the Vmax of the system could be found.

DISCUSSION

The results presented here indicate that the catalytic element dph, carrying 3ChA degradation information, is a viable genotype in a natural aquatic community. The sub-strain 3ChA acts as a strong selective force, and at micromolar level concentrations the rapid establishment of dph-harborinig organisms from inocula as small as that apparently provided by aerosol contamination. The genotype is selected for in a natural system in proportion to the chlorobenzene added to the system and remains active even though the original inoculum loses culturability on selective media.

Selection for the dph genotype became noticeable only after chlorobenzene concentrations reached 1 μM or more. Below that concentration, both the numbers of probe-positive organisms and the Vmax for the community were not significantly different from those found in microcosms lacking chlorobenzene inputs. Schön and Alexander (21) have theorized that below a certain threshold concentration, a particular substrate cannot support the growth of cells catalyzing it. They place this threshold at around 2 μg/l or approximately 10 nM of a typical simple aromatic com-

Fig. 3. Relationship between average log probe-positive (PROBE +) organisms and log[3ChA feed concentrations] in microcosm waters in 1988 (A; r² = 0.56, y = 0.475x + 3.65) and for sediments (O, r² = 0.59, y = 0.555x + 4.40).

Fig. 5. Relationship between log[Vmax] and log[probe-positive organisms] in microcosm waters. For 1987, r² = 0.80; y = 0.697x - 1.20. For 1988, r² = 0.69; y = 0.499x - 0.9.
pound. Janke (8) has argued that growth requirements may be the primary consideration for compounds degraded by constitutive enzymes but the concentration required for induction of enzyme synthesis is more important for other compounds. He found a phenol-oxidation induction threshold of 2 µM of the inducing compound. Similarly, induction of chlorobenzene catabolism in an Actinomadura celulace-
us strain occurred at comparable levels (16). Our data suggest that chlorobenzene does not select for the dph genes below 1 µM. This supports the idea that the induction threshold is more relevant to the survival of catabolic bacteria than a growth threshold.

Chlorobenzene was not the only determinant of the survival of the dph genes in the microcosms. Probe-positive organisms, presumably carried by aerial spread to and reproduced in microcosms receiving no 3Cha inputs in the 1987 initial release experiments. After introduction to micro-

ocisms with a final range of 3Cha concentrations in 1988, BR60 was seen to flourish in four of the microcosms independent of their 3Cha inputs. After 3 weeks, positively hybridizing organisms existed in 6 of the microcosms, that receiving 11 nM 3Cha and that receiving 10.9 µM 3Cha, at levels much higher than would be predicted from their 3Cha concentra-
tions alone (Fig. 3). Being adjacent to one another, these two microcosms likely received similar batches of sediments from a heterogeneous sediment source at the start of the experi-
ment. An unidentified alternate substrate or nutrient may have enhanced the survival of probe-positive organisms in these two microcosms. Other genes carried either on the pBR60 plasmid or on the chromosome of BR60 may have determined the fitness of this strain in these particular microcosms.

3Cha uptake rates in the microcosms were best predicted by the contaminant exposure levels. This result is new (18). What is new is the strong association between the heterotrophic potential and the population of bacteria carrying a specific genotype in this system. The relationship was not observed using probe-negative bacteria in the two experiments (Fig. 5) demonstrates that the MPN-DNA hy-
bridization technique gives a meaningful estimate of the 3Cha-degrading population. We obtained two different pre-
dictive relationships: the microcosm populations sampled in 1987 of MPN-DNA hybridizing organisms than those sampled in 1988. There are three possible reasons for this. First, the slope of the 1987 relationship could be artifically high because some of the higher probe-positive estimates are minimum estimates only. Second, the overall nutrient levels in the 1988 microcosms would have been reduced by the 130-µm filter in the water intake. A significant portion of the biodegradable organic matter that would normally be attacked by the bacterial flora and recolored as labile carbon, nitrogen, and phosphorus was removed from the system by this filter. Third, the dilution rate in the 1987 microcosms averaged 0.65%/hr was reduced to 0.01/hr in the 1988 experiment. The lower dilution rate would have selected for a population with a lower growth rate in the 1988 experiment.

The exact nature of the probe-positive organisms remains unclear. There were discrepancies between probe-positive estimates derived from general and selective media in 1987. Colonies failed to form on 3Cha plates in 1988 when posi-
tively hybridizing organisms were abundant (Fig. 2). These two observations indicate that probe-positive genes detected were carried by organisms cultivable in general liquid me-
dium but not cultivable on 3 nM 3Cha plates or in 3Cha liquid media. Bacteria fitting this description might include

BR60 cells with an altered 3Cha metabolism or bacteria other than BR60. There is evidence for both of these possibilities.

First, phenotypic variation in 3Cha metabolism was evi-
dent in BR60 colonies on 3Cha plates; many colonies accu-

mulated the toxic products of chlorobenzene degradation, a toxic metabolic intermediate not accumulated in high 3Cha-
adapted populations. Chlorobenzene accumulation on 3Cha agar plates or a complete loss of culturability on 3Cha plates could be a manifestation of a genotypic or phenotypic adaptability to low 3Cha levels encountered in the micro-

ocisms. Both phenotypic and genotypic changes have been documented in strains adapting to new substrate regimens (14, 15).

Second, pBR60 is a conjugative plasmid that transfers at a high frequency under laboratory conditions with conspecific strains. Its full host range is not known, but DNA probing of samples from Bloody Run Creek in the Niagara region (the site of origin of BR60) demonstrated a high frequency of probe-positive organisms, among them a Pseudomonas aci-
doverdant carrying pBR60 (24, R. R. Fulthorpe, R. C. Wynd-
ham, and N. A. Strous, Proceedings of the 11th Aquatic Toxicology Symposium, Special Tech. Pub. 2007, in press). Strains isolated from microcosms during these experiments have been found that differ from BR60 but carry pBR60 and exhibit 3Cha catabolism.

Studies are under way to determine which of these two possible sources of probe-positive bacteria determine the bulk of the probe-positive bacteria-viable BR60 discrepancy in the environment. Probe-positive estimates account for most of the variation in the 3Cha uptake rates, while the viable BR60 counts cannot. We already know that these extra probe-positive organisms play an active role in 3Cha degradation, but we cannot say if they are predominantly composed of adapted BR60 or new hosts of the dph genes. Not only the variance in 3Cha uptake rates was explained by our MPN-DNA probe-positive estimates. The possibility remains that the higher probe-positive estimates are not culturable even on general liquid media and therefore are not detectable by the MPN-DNA hybridization tech-

nique. Techniques not dependent on initial culturing of the sample, such as the Taq polymerase chain reaction (22) are being developed for tracking of environmental samples. Some of the variation in 3Cha uptake rates not ex-

plained by our probe-positive count might be accounted for by populations of truly nonculturable dph-harboring bacte-
ria. Nevertheless, the dependence of chlorobenzene uptake rates on the concentration of dph-carrying populations as determined by the MPN-DNA hybridization method, lends credence to the expectation that catabolic gene frequencies are a meaningful measure of biodegradative potential in a natural system. The predictive value of this measurement would be most useful in systems in which the contaminant exposure history, to date still the best predictor of biodeg-
rative potential, is unknown.

Jain et al. (7) examined the persistence of plasmid-bearing bacteria in ground aquifer material and concluded that catabolic plasmids can be maintained in a microbial commu-
nity in the absence of any known selection pressure. We have examined the persistence of an untaxed catabolic element, rather than an entire plasmid, in a highly realistic setting. In experiments described here, probe-positive or-

organisms were eliminated from the sediments of microcosms with no 3Cha in the 1987 release experiment by the end of 8 weeks. In the 1988 experiment, sediment probe-positive populations had declined to 10⁴ cells per ml in three of the
few microcosms receiving less than 70 nm SCMax for 3 weeks. In the absence of known selection, the likelihood of the dpl element being lost from the system is high but not a foregone conclusion. The higher population of probe-positive organisms in microcosms B1 (1 nm feed; Fig. 5) is evidence that other selective factors are important. A separate microcosm set up in 1988 in a different building approximately 500 m from the toxicology facility was doped with high levels of SCMax and developed a healthy population of streptomycin-sensitive B60 in 1988. The most likely source of this population was the sediments collected from the lake and used in the microcosms. This would imply that the streptomycin-sensitive strain used in release experiments in 1987 survived the winter in the sediments but was not detected by the MPN-DNA probe hybridizations the next spring.

For their rapid persistence studies, Jain et al. (7) used sealed glass vials containing aquifer materials as microcosms. These were stored at a constant temperature and closed to nutrient outflows. Our experimental microcosms at Lake Opicon provided a more rigorous test of the fitness of a very specific DNA element in a natural system. Organisms carrying the dpl genes were under constant pressure to compete with incoming lake microflora, to adapt to changing nutrient conditions, diurnal temperature changes, and the predators and toxins associated with lake ecosystems. The dilution rate used challenged the organisms to grow or obtain refuge in the sediments at the washed out of the microcosm waters. In the absence of selection, the population of the probe-positive organisms was to drop to extremely low levels but not low enough to be considered eliminated from the system.

Finally, this study showed that viable counts are clearly an unreliable method of determining the survival of this particular catabolic genotype in a natural system. Studying relying on plate counts for the investigation of specie survival should be reevaluated in light of these results (12). The phenotypic plasticity of bacterial strains and their capacity for genetic transfer must be considered. This is of particular importance when the unit of interest is not a bacterial strain but a gene sequence that it carries.

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