Survival of Pseudomonas putida UWC1 Containing Cloned Catabolic Genes in a Model Activated-Sludge Unit

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Received 21 February 1989/Accepted 6 July 1989

The possibility of the accidental or deliberate release of genetically engineered microorganisms into the environment has accentuated the need to study their survival in, and effect on, natural habitats. In this study, Pseudomonas putida UWC1 harboring a non-self-transmissible plasmid, pD10, encoding the breakdown of 3-chlorobenzoate was shown to survive in a fully functioning laboratory-scale activated-sludge unit (ASU) for more than 8 weeks. The ASU maintained a healthy, diverse protozoa population throughout the experiment, and the introduced strain did not adversely affect the functioning of the unit. Although plasmid pD10 was stably maintained in the host bacterium, the introduced strain did not enhance the degradation of 3-chlorobenzoate in the ASU. When reisolated from the ASU, derivatives of strain UWC1(pD10) were identified which were able to transfer plasmid pD10 to a recipient strain, P. putida PaW340, indicating the in situ transfer of mobilizing plasmids from the indigenous population to the introduced strain. Results from plate filter matings showed that bacteria present in the activated-sludge population could act as recipients for plasmid pD10 and actively expressed genes carried on the plasmid. Some of these activated-sludge transconjugants gave higher rates of 3-chlorobenzoate breakdown than did strain UWC1(pD10) in batch culture.

The genetic manipulation of aromatic catabolic pathways in bacteria is an important method for achieving improved rates of degradation of recalcitrant xenobiotic compounds (42, 45, 51). This approach has resulted in the construction of recombinant bacteria capable of novel activities in pure culture, but little work has been done to test survival and degradation rates under environmentally relevant conditions, such as those existing in wastewater treatment works. Several studies have investigated the persistence of genetically engineered microorganisms (GEMs) in natural or model ecosystems such as wastewater treatment plants, activated-carbon filters, fresh water, and soil (7, 15, 40, 43, 44, 47, 52).

Although bacteria introduced into complex ecosystems may rapidly decline in number, enhanced degradation rates might be achieved by transfer of degradative genes to the natural flora. Gentner et al. (22) demonstrated the capacity of natural freshwater isolates to act as recipients of the broad-host-range plasmids R68 and R1162. Kolenc et al. (29) also demonstrated that a psychrotrophic bacterium, Pseudomonas putida Q5, acted as a recipient for the TOL plasmid pWW0 and expressed the pWW0-encoded catabolic activities at low temperatures. Until recently, most investigations into gene transfer in natural aquatic systems have used membrane diffusion chambers or dialysis sacs, with Escherichia coli as the host bacterium (1, 31). Most of these studies excluded the indigenous microflora, although plasmid transfer in the presence of natural bacteria in aquatic test chambers has been demonstrated (10, 36).

This study was designed to investigate the survival of a 3-chlorobenzoate (3CB)-degrading bacterium, P. putida UWC1 harboring a recombinant plasmid, pD10, in a laboratory scale activated-sludge unit (ASU).

MATERIALS AND METHODS

Bacterial strains and plasmids. P. putida UWC1 is a spontaneous rifampin-resistant derivative of P. putida KT2440 (4). Recipient strains used in matings were Alcaligenes eutrophus JMP222 (Smr [16]), E. coli J53 (Rif’ [3]), and a derivative of the tryptophan auxotroph P. putida PaW340 (Trp’ Smr’ [26]), into which the tetracycline resistance determinant of TnJ0 was introduced by using the suicide vector pLG223 (9), designated strain UWC2.

Plasmid pD10 (3CB+ Km’ [see below]) is a recombinant plasmid consisting of the Smr C fragment of plasmid pJP4, which encodes the essential steps in chlorocatechol catabolism (16), cloned into the broad-host-range vector pKTC213 (4). This plasmid enables P. putida UWC1 to utilize 3CB as the sole source of carbon and energy. Isolated natural bacteria were identified by using the API 20NE identification system (API Laboratory Products Ltd., Basingstoke, United Kingdom).

Media for growth and maintenance of bacteria. Bacterial strains were maintained on L agar plates (35) containing the appropriate antibiotics. The minimal medium used for obtaining growth on defined carbon sources was as previously described (46). King B medium (28) was used to confirm the identity of fluorescent Pseudomonas spp. Concentrations of antibiotics used were as follows: kanamycin, 50 μg/ml; rifampin, 100 μg/ml; tetracycline, 100 μg/ml.

Laboratory ASU. The ASU consisted of a 4 liter perspex aeration chamber (19 cm by 19 cm by 12.5 cm) with a liquid overflow to a 1-liter tubular settling tank (diameter, 7 cm; height, 32 cm). The settled sludge was returned to the aeration chamber by a peristaltic pump, and the clarified effluent was removed by a liquid overflow to a waste vessel. The mixed liquor in the aeration chamber was aerated (at 250 ml/min) with an air pump and sintered-glass block and constantly agitated (at 100 rpm) by a mechanical stirrer. The feed consisted of wastewater collected from the influent of a domestic wastewater treatment works (Cynon Valley, South Wales). This was sterilized by autoclaving and supplemented with artificial sewage, which contained the following (in milligrams per liter): Bacto-Peptone (no. 0118.01.8; Difco Laboratories, East Molesey, United Kingdom), 160; beef extract (no. 1871-17; Difco), 110; urea, 30; NaCl, 7;
CaCl·2H₂O, 4; MgSO₄·6H₂O, 2; K₂HPO₄, 23. The feed between days 22 and 27 consisted of nonsterile supplemented wastewater, and from day 31 onward 3CB was introduced into the unit either as a single shock load directly into the aeration chamber (100 mg/liter on days 31 and 33; 500 mg/liter on day 35) or, from day 40, as a constant input (1,000 mg/liter) with the feed. The residence time was 24 h, and the sludge wastage was 10% per day. The unit was insulated and operated at ambient temperature (the temperature ranged from 8 to 22°C over the initial 8-week period of the experiment). After day 56 the temperature of the unit was maintained at a constant 15°C by using a chiller thermocirculator (Churchill Instrument Co. Ltd., Perivale, United Kingdom).

*P. putida* UWC1(pD10) was grown for 18 h in L broth with kanamycin (40 ml) and harvested by centrifugation at 6,000 × g at 4°C for 10 min (RC5C centrifuge; Ivan Sorvall, Inc., Norwalk, Conn.). The cell pellet was suspended in 20 ml of the ASU feed (sterile supplemented wastewater), and the suspension was then inoculated directly into the mixed liquor.

The performance of the unit and sludge settlement were monitored by measuring the biochemical oxygen demand (BOD), suspended solids, mixed-liquor suspended solids, and sludge volume index by standard methods (2). Protozoal species were identified and counted by phase-contrast microscopy of the settled sludge. Aliquots of settled sludge (30 μl) were placed under a glass cover slip (1 cm by 1 cm), and the number of protozoa in 50 random fields of view were counted. Protozoa were identified on the basis of anatomical characteristics by using a key specific for ciliated protozoa found in activated sludge (11).

**Bacterial counts.** Total bacterial counts in the mixed liquor were estimated by using acridine orange staining followed by epifluorescence microscopy essentially as previously described (20), except that filters were stained with Irgalan black (0.25%). Sludge flocs were dispersed by homogenization in the presence of the defloculants cissarol ALN-WF, formerly Lubrol W (0.01%, wt/vol; ICI Organics, Macclesfield, United Kingdom), and sodium PE (0.01%, wt/vol [21]) by using an IKA ultra-turrax (no. TP18/10; Sartorius Instruments Ltd., Belmont, United Kingdom) followed by gentle sonication for three 1-min intervals at 1.5 A in a 60-W ultrasonic disintegrator (No. 5000; M.S.E., Crawley, United Kingdom).

Viable counts of heterotrophic bacteria were estimated on casitone-glycerol-yeast extract medium (CGY [37]). Bacteria were enumerated by the method of Miles and Misra (34).

**Identification of GEM in activated sludge.** Three different media were used for enumeration of the recombinant strain UWC1(pD10) in the mixed liquor of activated-sludge samples. These media were chosen because they were more efficient than other media tested for recovery of the introduced strain and counterselection against the natural sludge microflora. The first was PsRF, a *Pseudomonas* selective medium (CM559; Oxoid Ltd., London, United Kingdom) containing supplements of cetrimide and nalidixic acid (SR102; Oxoid) and rifampin (100 μg/ml). The host strain UWC1, whether or not it harbored plasmid pD10, was enumerated on this medium. The second was LKmRF, which consisted of L agar containing kanamycin (50 μg/ml) and rifampin (100 μg/ml). The host strain UWC1, whether or not it harbored plasmid pD10, was enumerated on this medium. The second was LKmRF, which consisted of L agar containing kanamycin (50 μg/ml) and rifampin (100 μg/ml). It was used to estimate the numbers of UWC1 retaining Km . The third was 3CBKm, which consisted of 3CB (3 mM) plus kanamycin (50 μg/ml). It was used to estimate the numbers of bacteria retaining or expressing the 3CB+ Km phenotype encoded by the recombinant plasmid, pD10. By using these three media, it was possible to detect 10⁵ CFU of strain UWC1 per ml against a background count on CGY of 10⁵ CFU/ml. CGY plates were incubated at 20°C for 48 h; all other plates were incubated at 30°C for 24 to 48 h before the colonies were counted. Bacteria present in the activated-sludge flocs were separated from the mixed-liquor supernatant before enumeration by centrifugation at 500 × g for 2 min in a Sorvall RC5C centrifuge.

**Conjugation experiments.** Plate filter matings were carried out by mixing 50 μl of fivefold-concentrated 18-h L broth cultures of donor and recipient strains on sterile membrane filters (type WCN; diameter, 25 mm; pore size, 0.45 μm; Whatman, Inc., Clifton, N.J.) placed on L agar plates. Control filters consisted of donor or recipient cultures alone. The filters were incubated for 6 to 8 h at 30°C, and the cells were then suspended in 2 ml of L broth by vortex mixing. Undiluted and serially diluted samples were plated onto selective media for detection of transconjugants and enumeration of donor cells. Transfer frequencies were expressed as the number of transconjugants per donor cell.

Multiple patch matings were carried out by growing the donor strain [UWC1(pD10) reisolated from the ASU on PsRF agar] on L agar for 24 h and transferring purified colonies to a lawn of recipient cells (strain UWC2) prepared from 100 μl of a twofold-concentrated 18-h L broth culture spread on a selective plate (consisting of L agar, streptomycin, tetracycline, and kanamycin). The recipient population was then used to detect plasmid transfer to indigenous activated-sludge bacteria in vitro consisting of 1 ml of filtered activated sludge (containing ca. 10⁸ CFU/ml on CGY). Transconjugants were selected on solid minimal medium containing 3CB (as the sole carbon and energy source) plus kanamycin. All transconjugants were purified on fresh selective plates and examined for phenotypic markers and plasmid content.

**Plasmid screening.** Plasmids were detected by a modification (41) of the method of Kado and Liu (27). Rapid, small-scale preparations of plasmid DNA for restriction endonuclease digestion were made by the method of Holmes and Quigley (24). Restriction endonucleases were obtained from New England Biolabs Ltd., Cramlington, United Kingdom, and were used as specified by the manufacturer.

**Chloride release.** The release of chloride accompanying 3CB metabolism was detected in the ASU and in batch cultures by using a Marius Chlor-o-Counter (F.T. Scientific Instruments Ltd. Gloucester, United Kingdom) (46) calibrated with a 50 mM NaCl standard. Free Cl⁻ ions were titrated against coulometrically generated Ag⁺ ions, resulting in precipitation of AgCl. Amperometric detection of the endpoint by silver electrodes coincided with the appearance of free Ag⁺ ions in the reaction vessel. The ability of strains to cataleobize 3CB was assessed by using 250-ml flasks, incubated at 30°C with agitation (100 rpm), containing 50 ml of wastewater supplemented with 500 mg of 3CB per liter and 5 ml of settled activated sludge. Chloride concentrations were measured daily (the maximum possible Cl⁻ was 3.9 mM).

**Statistical methods.** Statistical analysis of the data was performed as described previously (5).

**RESULTS AND DISCUSSION**

**Performance of the ASU.** The ASU was allowed to stabilize for 24 days following inoculation with fresh activated
sludge. This allowed acclimatization to the feed and the establishment of a stable sludge population. Close monitoring of the stabilized ASU, prior to inoculation with strain UWC1(pD10), provided control data for the continuous operation which were compared with data obtained postinoculation. During the acclimatization period and throughout the experiment, the performance of the ASU was monitored and found to achieve consistently large reductions in BOD and suspended solids (Table 1). There was a gradual reduction in the mixed-liquor suspended solids, although the sludge volume index, which was a measure of the rate of settlement of the sludge, remained fairly constant, indicating a sludge with good settling characteristics (sludge volume index = 200).

The total numbers of protozoa in the settled sludge were stable throughout the experiment (Table 2). Protozoa play an important role in the activated-sludge process (12, 13). Data from both laboratory scale and full-scale plants show that in the absence of a healthy protozoal population, highly turbid effluents of poor quality are produced (12). Protozoa can also be used as biological indicators of the performance of an ASU (14, 38). Good activated sludges generally contain protozoal populations dominated by attached and crawling ciliates, with small numbers of flagellates and amoebae. The protozoal populations observed during this study were dominated by attached ciliates, especially Vorticella spp.; crawling and free-swimming protozoa such as Aspidisca costata and Trachelophyllum pusillum were observed throughout the experiment. Several of the protozoal species recorded (e.g., Vorticella campanula and Euplotes affinis) are commonly

### TABLE 1. Variation of BOD, suspended solids, and sludge volume index in the laboratory scale ASU before and after addition of *P. putida* UWC1(pD10)

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>BOD (mg of O₂/liter)</th>
<th>Reduction in BOD (%)</th>
<th>Suspended solids (mg/liter)</th>
<th>Reduction in suspended solids (%)</th>
<th>Mixed-liquor suspended solids (mg/liter)</th>
<th>Sludge volume index</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Influent</td>
<td>Effluent</td>
<td>Influent</td>
<td>Effluent</td>
<td>Influent</td>
<td></td>
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<td>27</td>
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<td>ND</td>
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<td>84</td>
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<td>40</td>
<td>86</td>
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</table>

Mean value:
- BOD: 227 mg O₂/liter
- Suspended solids: 91 mg/liter
- Sludge volume index: ND

CV = 4.5%

MSR = 161

ND = Not determined

* UWC1(pD10) was introduced into the unit on day 0.
* Mean value of two replicate determinations.
* ND, Not determined.
* CV, Coefficient of variation (%).
* MSR, Minimum significant range.

### TABLE 2. Numbers of ciliated protozoa in the laboratory scale ASU settled sludge before and after addition of *P. putida* UWC1(pD10)

<table>
<thead>
<tr>
<th>Time (days)</th>
<th><em>A. costata</em></th>
<th><em>T. pusillum</em></th>
<th>Other free swimmers</th>
<th><em>V. convallaria</em></th>
<th>Other attached solitary organisms</th>
<th>Attached colonial organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1</td>
<td>2.3 × 10⁴</td>
<td>2.4 × 10⁷</td>
<td>8.0 × 10²</td>
<td>1.1 × 10⁴</td>
<td>1.2 × 10⁴</td>
<td>1.7 × 10³</td>
</tr>
<tr>
<td>6</td>
<td>1.1 × 10⁴</td>
<td>1.1 × 10⁷</td>
<td>2.8 × 10³</td>
<td>2.6 × 10⁴</td>
<td>4.8 × 10⁴</td>
<td>1.5 × 10⁵</td>
</tr>
<tr>
<td>13</td>
<td>2.6 × 10⁴</td>
<td>1.1 × 10⁷</td>
<td>ND</td>
<td>2.7 × 10⁴</td>
<td>2.8 × 10⁴</td>
<td>6.6 × 10⁵</td>
</tr>
<tr>
<td>21</td>
<td>2.7 × 10⁴</td>
<td>1.7 × 10⁷</td>
<td>7.0 × 10³</td>
<td>2.2 × 10⁴</td>
<td>1.5 × 10⁴</td>
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<td>8.0 × 10³</td>
<td>3.8 × 10⁷</td>
<td>1.1 × 10⁴</td>
<td>5.5 × 10⁴</td>
<td>1.6 × 10⁴</td>
<td>2.0 × 10⁷</td>
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<tr>
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<td>4.7 × 10³</td>
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<td>ND</td>
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<td>2.8 × 10⁴</td>
<td>1.5 × 10⁵</td>
</tr>
<tr>
<td>50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>8.0 × 10³</td>
<td>2.5 × 10⁴</td>
<td>1.7 × 10⁷</td>
</tr>
<tr>
<td>55</td>
<td>1.2 × 10³</td>
<td>ND</td>
<td>ND</td>
<td>2.5 × 10⁴</td>
<td>2.9 × 10⁴</td>
<td>1.9 × 10⁷</td>
</tr>
</tbody>
</table>

* Addition of UWC1(pD10) on day 0. First addition of 3CB on day 31.
* Other species identified (but numbers below 10⁵/ml): *V. campanula, V. microstoma, V. ocellata, V. striata, Podophyra maupasi* (attached solitary); *Opalularia minima, O. coarctata* (attached colonial); *Chilodonella uncina*, *E. affinis* (free swimming or crawling).
* ND, Not detected (limit of detection, 1 × 10⁴ protozoa per ml).
associated with activated-sludge treatment works producing high-quality effluent (14). After day 35, following the introduction of 3CB into the feed, there was a decline in the numbers of T. putidum. Over the same period, there was a noticeable increase in the numbers of colonial attached peritrichs. Similar variations in the numbers of individual species in full-scale units have been observed elsewhere (39). The protozoal species identified in the ASU (Table 2) were characteristic of an activated-sludge plant producing a high-quality effluent (BOD, 11 to 20 mg/liter), on the basis of the species association rating of Curds and Cockburn (13). This correlates well with the average effluent BOD of 20 mg/liter observed in our experiment (Table 1). The presence of a healthy, diverse protozoal population in the ASU throughout the experiment further demonstrated that the introduced strain, UWC1(pD10), did not have a deleterious effect on the performance of the unit or on the higher orders of organisms present.

**Survival and growth of UWC1(pD10) in the ASU.** P. putida UWC1(pD10) was inoculated into the ASU mixed liquor at 4 x 10^6 CFU/ml on day 0, and the survival of the host strain and recombinant plasmid were separately monitored in the absence (days 0 to 30) and subsequently in the presence (days 31 to 70 [Fig. 1]) of added 3CB. The viable counts on the three selective media (LKmRF, PsRF, and 3CBKm) were not significantly different (Fig. 1), suggesting that the plasmid was stably maintained in the host strain. Individual colonies growing on PsRF plates were also purified and tested for kanamycin resistance and the ability to utilize 3CB as the sole carbon and energy source (3CB^). The colony morphologies of all isolates obtained on these media were indistinguishable from that of the introduced strain; all colonies patched onto King B medium produced fluorescent pigment. Before day 45, all colonies growing on PsRF plates were also kanamycin resistant and 3CB^ (≥96 colonies tested at each 7-day interval). From samples taken on days 45, 49, and 56, 89%, 96%, and 97.5%, respectively, of the colonies arising on PsRF plates were also kanamycin resistant and 3CB^+. All of the isolates tested (12) from PsRF medium on day 56 grew rapidly on 3CB in liquid culture and dechlorinated this substrate completely. Plasmids purified from each of these were found to be indistinguishable from pD10 on the basis of digestion with the restriction endonuclease SstI (data not shown). These results clearly confirmed the specificity of the selective media used to reisolate strain UWC1(pD10).

The introduced strain persisted in the ASU, in the presence of a heterogeneous sludge microflora containing predatory protozoa, for more than 8 weeks, although the population size declined gradually. Other workers have shown that in the presence of competitive natural microbial flora in sewage and river water, E. coli populations declined rapidly (18). Another study showed that in the presence of an autochthonous microflora, the survival of E. coli, P. aeruginosa, and P. putida on activated-carbon filters was adversely affected (43). By contrast, the results of Jain et al. (25) and Van der Meer et al. (49) indicated that Pseudomonas spp. are resistant to starvation and can survive for long periods in model ecosystems containing natural populations.

The decline in numbers of strain UWC1(pD10) was multiphasic (Fig. 1). There was an initial rapid decline (days 0 to 12), at a rate similar to that expected as a result of dilution with fresh medium. This was followed by a period when there was no significant reduction in the numbers (days 12 to 28), and the slope of the regression line with time was not significantly different from zero. To maintain a viable population for this length of time, growth of the introduced organism within the sludge flocs must have occurred. Plate counts indicated that during the period of stabilization (days 12 to 28), more than 90% of the recombinant bacteria were in the sludge flocs. From days 28 to 56, there was a gradual but significant decline of the introduced population at a rate similar to the sludge wastage rate. The ability of an introduced strain to flocculate and become incorporated into activated-sludge flocs is an important characteristic if it is to contribute to the biodegradation of xenobiotics in the influent. Return with the settled sludge would aid persistence in the unit, and the bacteria would be protected by the sludge flocs from predatory protozoa, which feed mainly on suspended bacteria.

The presence of 3CB in the ASU feed did not appear to halt the decline in numbers of the introduced strain. In addition, over the period in which nonsterile supplemented wastewater was used as the feed, there was no significant reduction in the numbers of strain UWC1(pD10). The total bacterial count remained constant throughout the operation of the unit (Fig. 1). The viable count on CGY was also constant, except for occasional small, significant decreases and a more noticeable drop 1 day after the first introduction of 3CB into the unit. However, the viable count recovered rapidly and was unaffected by further introduction of 3CB.

On day 58 the unit was reisolated with the same titer (ca. 4 x 10^6 CFU/ml) of P. putida UWC1(pD10) (Fig. 1) to assess whether the rapid decline, observed after the first inoculation, was affected by the presence of 3CB. This second period of decline was shorter (3 days) than that observed previously (10 days). The population of the introduced strain also stabilized at a higher level (ca. 2 x 10^7 CFU/ml) than previously (ca. 2 x 10^6 CFU/ml).

**Acquisition of mobilization functions by P. putida UWC1(pD10) in situ.** Plasmid pD10 is not self-transmissible but, being a derivative of the vector pKT231, can be mobilized by certain conjugal plasmids present in the same cell, e.g.
IncIα and IncP1 plasmids (19). Colonies of *P. putida* UWC1(pD10), reisolated from the ASU mixed liquor on PsRF plates, were tested for their ability to transfer the plasmid-borne kanamycin resistance determinant to *P. putida* UWC2 (3CB− Trp− Sm− Tc+) in multiple patch matings. Before day 36, none of the reisolated UWC1 strains tested could transfer kanamycin resistance to strain UWC2, but from day 36 onward, several such strains were isolated. The maximum proportion of strain UWC1(pD10) reisolated from the ASU which could transfer kanamycin resistance to strain UWC2 was detected on day 49 (6 of 192). All putative transconjugants tested (10) were auxotrophic, tetracycline resistant, and able to utilize 3CB in the presence of tryptophan, indicating that plasmid pD10 had been transferred intact from UWC1 to UWC2. Isolation of plasmids from these transconjugants, designated UWC2.R1 to UWC2.R10, confirmed that they had all acquired a plasmid of similar size to pD10 and that at least two of the transconjugants, strains UWC2.R1 and UWC2.R5, had also acquired larger plasmids (data not shown). On the basis of these results, it is suggested that the ability of some UWC1(pD10) derivatives isolated from the ASU to transfer plasmid pD10 to strain UWC2 resulted from their having acquired mobilization functions and/or plasmids from the sludge bacteria. Hence, there was clear evidence for genetic interactions between the GEM and the sludge microflora.

To confirm the presence of mobilizing plasmids in strain UWC2(pD10) transconjugants, we carried out filter matings by using strain UWC1 (3CB− Rif+) as the recipient. Transfer of the plasmid pD10 kanamycin resistance determinant and catabolic genes (encoding the 3CB− phenotype) from strain UWC2.R5 (3CB+ Trp− Sm− Tc+) occurred at a frequency of 8.8 × 10−4 per donor cell. Transconjugants were selected on medium containing 3CB (as the sole carbon and energy source) and kanamycin. Other UWC2-derived strains tested (UWC2.R1 to UWC2.R4) were also found to mobilize plasmid pD10. In addition, strain UWC2.R5 could transfer kanamycin resistance to *E. coli* J53 (Rif+) and *A. eutrophus* JMP222, suggesting that the mobilizing plasmid in this strain encoded broad-host-range transfer functions.

Other investigators have presented results which suggest that plasmid transfer in sewage and aquatic systems occurs in the presence of a competitive natural population (6, 44, 48). Derivatives of strain UWC1(pD10) which were transfer proficient (Tra+) were reisolated from the ASU on PsRF plates, which should not preferentially select for Tra+. Thus, it is likely that the introduced strain received mobilizing plasmids from the indigenous wastewater population in the ASU. As no Tra+ strains were detected in the ASU before day 36, the acquisition of mobilization functions and/or plasmids was probably a rare event. In the aeration chamber of the ASU, which was constantly agitated, the sludge flocs represented areas of high bacterial concentration, favoring conjunctive-pair formation. McPherson and Gealt (33) described the mobilization of indigenous wastewater bacterial strains capable of mobilizing plasmid pBR325, and Mancini et al. (32) showed the mobilization of plasmid pHSV106 in a laboratory scale waste treatment facility by using both laboratory and indigenous strains of the mobilizer and recipient. Although the latter study was conducted in the absence of a heterogeneous wastewater population, results from these studies and those presented in this paper indicate a high potential for transfer of non-self-transmissible plasmids between introduced strains and natural wastewater bacteria.

**Plasmid transfer to indigenous activated-sludge bacteria in vitro.** Strains UWC2.R2 and UWC2.R5 (Trp− 3CB− Km− derivatives of strain UWC2 into which plasmid pD10 had been mobilized) were used as donors in in vitro filter matings, with the recipient population consisting of filtered activated sludge. Control plates gave no 3CB+ prototrophs (selection on 3CB plus kanamycin). A total of 28 transconjugants (designated ASR2.1 to ASR2.14 from matings with UWC2.R2 as the donor and ASR5.1 to ASR5.14 with UWC2.R5 as the donor) were isolated; the maximum transfer frequency observed was 3.5 × 10−7 per donor. All transconjugant strains tested were gram negative and oxidase positive, and 11 of the strains produced fluorescent pigment on King B medium and were therefore designated fluorescent *Pseudomonas* spp. These filter-mating experiments, conducted with natural activated-sludge bacteria as a recipient population, indicated that strains were present in the indigenous microflora which were suitable hosts for plasmid pD10 and which efficiently expressed catabolic and resistance genes carried on the plasmid.

Figure 2 shows the plasmid profiles of 11 of the transconjugants selected at random; the screening indicated that most, if not all, of the 28 transconjugants carried a plasmid of similar size to pD10. Digestion of plasmid DNA from the transconjugants confirmed that the recipients in the activated sludge had received plasmid pD10 (Fig. 3). The 13-kilobase vector band and the 7-kilobase insert band derived from plasmid pJP4, produced by SalI digestion of plasmid pD10, were apparent in all the strains except ASR5.1 and ASR5.6 (Fig. 3, lanes H and M, respectively), from which no plasmid DNA could be extracted by the method of Holmes and Quigley (24). This rapid method of extracting plasmid DNA suitable for restriction endonuclease digestion was developed for *E. coli* and is not generally applicable to other genera, although the method also works well for many *P. putida* strains.

On the basis of colony morphology, fluorescence on King B medium, and plasmid content, the activated-sludge transconjugants were divided into 19 distinct groups for further analysis and identification.

**Chloride release in the ASU.** No significant Cl− release above the endogenous levels present in the influent was measured in the effluent from the ASU up to day 56. The introduced strain, UWC1(pD10), retained the ability to grow on 3CB, but no breakdown of this substrate was apparent in
the unit. Samples of mixed liquor taken on day 56 and shaken as batch cultures indicated total mineralization of 3CB after a lag period of 4 to 6 days. These data suggested that the potential for 3CB breakdown was present in the mixed liquor but was not observed under normal plant-operating conditions at the GEM population levels present in the ASU. Chloride release (20 to 26% of the maximum possible) was detected in the ASU effluent on days 70 and 71, 11 days after inoculation with strain UWC1(pD10) (data not shown).

After day 72, the ASU was run in a batch mode with continued aeration and recirculation of sludge, but without fresh influent. After 7 days, degradation of the 3CB in the unit was indicated by the near-maximal release of Cl\(^-\) (893 mg/liter). However, dilution plate counts on 3CBKm and pSRF indicated that the predominant 3CB strain in the unit was a novel isolate, designated strain AS2. This strain flocculated readily in complex media and contained a 3CB-degrading plasmid unrelated to pD10 (data not shown); it is currently undergoing further characterization.

**Chloride release by 3CB-degrading strains in batch culture.**

The reasons for the failure of microbial inoculants to enhance biodegradation have been discussed by Goldstein et al. (23), who suggested that low substrate concentrations, predation, susceptibility to toxins, and preferential utilization of alternative substrates are possible problems in detoxification of specific pollutants. Dwyer et al. (17) described the survival of derivatives of *Pseudomonas* sp. strain B13 in a simple aerobic sludge ecosystem. Although one such derivative, strain FR1(pFRC20P), totally degraded combinations of 3CB and 4-methylbenzoate in pure culture, it was unable to do so in the sludge ecosystem, possibly because of the availability of other preferred growth substrates. Blackburn et al. (8) also showed that the degradation of naphthalene in activated sludge was dependent on the total numbers of naphthalene-degrading strains present.

The inability of strain UWC1(pD10) to mineralize 3CB in the ASU may have been due to the small numbers of 3CB\(^-\) strains present or the availability of other growth substrates. This possibility was examined by inoculating batch cultures containing supplemented wastewater and 500 mg of 3CB per liter with strain UWC1(pD10) at 4 \times 10^6 CFU/ml. Replicate flasks were set up and inoculated with strain AS2 and the 19 representative sludge transconjugants which had acquired the ability to grow on 3CB from strains UWC2.R2 or UWC2.R5 (i.e., strains ASR2.1 to ASR2.14 and ASR5.1 to ASR5.14). Figure 4 shows the rates of Cl\(^-\) release by strain UWC1(pD10), strain AS2, and six of the sludge recipient bacteria. Strain UWC1(pD10) exhibited a lag period of 3 days before any Cl\(^-\) was detected in the culture supernatant. The majority of the sludge-derived bacteria (15 of 19) showed a similar pattern, i.e., no Cl\(^-\) release detected before day 4 followed by rapid and total 3CB breakdown by day 7. Typical examples of this pattern of breakdown were observed in cultures inoculated with strains ASR5.4 and ASR5.14 (Fig. 4). These results suggested that 3CB degradation occurred only after other available carbon sources had been utilized. Alternatively, growth of strains which exhibited a lag period before dechlorination of 3CB may have been inhibited by the substrate.

Strain AS2 did not show a lag period before Cl\(^-\) release was detected, and maximum Cl\(^-\) release occurred after only 5 days. The enrichment of strain AS2 in the unit may have occurred because it catabolized 3CB more efficiently than the other strains in the wastewater. Sludge transconjugant strain ASR2.8 showed a similar pattern and rate of Cl\(^-\) release. The three strains which showed the most rapid breakdown of 3CB in the presence of supplemented wastewater and activated sludge were the sludge transconjugants ASR2.2 (identified as a *Pseudomonas* sp.), ASR5.2 (*P. putida*), and ASR5.10 (*P. putida*). The last two strains showed immediate and rapid release of Cl\(^-\) in batch culture. These preliminary results suggest that some of the natural bacterial strains containing plasmid pD10 may degrade 3CB more efficiently than strain UWC1(pD10) in aerobic waste treatment units.

This study has shown that a GEM, *P. putida* UWC1 (pD10), was easily detected and survived for more than 8 weeks after introduction into a laboratory scale ASU. Liang et al. (30) showed that bacteria varied in their ability to survive in sewage and lake water. Jain et al. (25) also showed that indigenous groundwater strains and *P. putida* harboring broad-host-range plasmids pWW0 (TOL) and RK2 were maintained at high levels for 8 weeks in a groundwater.
The ASU proved a very accurate model of out more efficient in situ degradation of xenobiotic compounds. On the basis of the physical and biological factors investigated, the ASU proved a very accurate model of out more efficient in situ degradation of xenobiotic compounds. The lack of significant change to the protozoan populations indicated that the introduction of the GEM used here did not adversely affect the complex biological community maintained in the unit. Such results provided potential information concerning the fate of GEMs in isolated ecosystems, as well as data which can be used to assess the possible environmental impact of GEMs: a prerequisite for the release of recombinant bacteria into the environment. In addition, the ASU provides a continuous-flow culture system in which strategic selection and/or gene transfer between indigenous and introduced recombinant bacteria may result in the isolation of strains which can carry out more efficient in situ degradation of xenobiotic compounds.

ACKNOWLEDGMENTS
We thank the Water Research Centre for supporting this research with a research contract and the award of the John L. van der Post fellowship to N.C.M.

LITERATURE CITED


