APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1991, p. 366–373
0099-2240/91/020366-08$02.00/0

Vol. 57, No. 2

Survival and Catabolic Activity of Natural and Genetically Engineered Bacteria in a Laboratory-Scale Activated-Sludge Unit

NICHOLAS C. McCLURE,* JOHN C. FRY, AND ANDREW J. WEIGHTMAN

School of Pure and Applied Biology, University of Wales College of Cardiff, P.O. Box 915, Cardiff CF1 3TL, United Kingdom

Received 5 June 1990/Accepted 4 November 1990

The survival of selected naturally occurring and genetically engineered bacteria in a fully functional laboratory-scale activated-sludge unit (ASU) was investigated. The effect of the presence of 3-chlorobenzoate (3CB) on the survival of *Pseudomonas putida* UWC1, with or without a chimeric plasmid, pD10, which encodes 3CB catabolism, was determined. *P. putida* UWC1(pD10) did not enhance 3CB breakdown in the ASU, even following inoculation at a high concentration (3×10^8 CFU/ml). The emergence of a natural, 3CB-degrading population appeared to have a detrimental effect on the survival of strain UWC1 in the ASU. The fate of two 3CB-utilizing bacteria, derived from activated-sludge microflora, was studied in experiments in which these strains were inoculated into the ASU. Both strains, AS2, an unmanipulated natural isolate which flocculated readily in liquid media, and *P. putida* ASR2.8, a transconjugant containing the recombinant plasmid pD10, survived for long periods in the ASU and enhanced 3CB breakdown at 15°C. The results reported in this paper illustrate the importance of choosing strains which are well adapted to environmental conditions if the use of microbial inoculants for the breakdown of target pollutants is to be successful.

Although considerable progress has been made in the isolation, selection, and manipulation of bacteria capable of degrading specific target pollutants, the application of this knowledge to practical wastewater treatment is still in its infancy. The potential and problems associated with the enhancement of wastewater treatment by the addition of microorganisms have been widely discussed (23, 33, 42). Few reports have described the successful use of microbial inoculants to enhance biodegradation in real wastewater treatment systems, although the inoculation of specific bacterial strains has been shown to have beneficial effects in both simple laboratory ecosystems and larger-scale oxygenated activated-sludge systems (14, 18, 21, 35, 39). However, few of these studies rigorously tested the survival and activity of the introduced organisms.

In recent years recombinant bacteria, especially *Pseudomonas* spp., with novel degradative capabilities have been constructed by using the techniques of genetic engineering. Unfortunately, little work has been done to assess the survival and in vivo activity of recombinant strains in wastewater treatment systems. There has been increasing interest in studies examining both the fate and effects of genetically engineered microorganisms that are released either deliberately or accidentally into natural ecosystems (3, 5, 25, 26, 37). Many of these studies have used contained laboratory microcosms to predict the persistence of genetically engineered microorganisms under environmentally relevant conditions and to examine interactions with indigenous microflora.

Recently, we described the survival of a genetically engineered bacterium, *Pseudomonas putida* UWC1(pD10), in a laboratory-scale activated-sludge unit (ASU) (26). The recombinant plasmid pD10 encoded three essential steps in the dissimilation of chlorocatechols, key metabolites in the breakdown of a number of chlorinated aromatic compounds, many of which are potential environmental pollutants (22). Although the introduced bacterium survived for more than 8 weeks in the activated-sludge ecosystem, no breakdown of the target substrate, 3-chlorobenzoate (3CB), was observed. We suggested that the absence of catabolic activity may have been due to biochemical interference (e.g., catabolic repression) resulting from the utilization of alternative carbon and energy sources by the introduced strain, and to the inability of the introduced strain to maintain an active, proliferating population at levels commensurate with the catabolism of 3CB within the ASU (say $>10^6$ CFU/ml). It was proposed, therefore, that a more effective inoculation strategy, aiming to establish 3CB-degrading activity in the ASU, would involve the use of microbial strains which were better adapted for survival and catabolism in this environment.

In this paper we describe the survival of both natural and recombinant bacterial strains in a model ASU and present evidence for enhanced breakdown of 3CB in situ by inoculation with activated-sludge-derived bacteria.

MATERIALS AND METHODS

Bacterial strains, growth, and enumeration. The bacteria used in this study are listed in Table 1. All strains were routinely maintained on Luria agar (L agar) at 30°C. Broth cultures were grown in L broth incubated at 30°C for 18 h in an orbital, shaking incubator. Viable bacteria were enumerated as described previously (26). Viable counts of heterotrophic bacteria in mixed liquor were made on CGY medium (31). Rifampin-resistant mutants of the unidentified gram-negative bacterium strain AS2 and *P. putida* ASR2.8 were selected for use as inoculants. The numbers of strain UWC1 were estimated by using *Pseudomonas* selective medium (PSRF, CM559; Oxoid Ltd., London, England) with a cetrimide-nalidixic acid supplement (SR102; Oxoid) and rifampin (100 μg/ml). L agar with kanamycin (50 μg/ml) and rifampin was used to enumerate *P. putida* ASR2.8, and L agar with rifampin alone was used to count strain AS2. Bacteria that degraded 3CB were enumerated by using a

* Corresponding author.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
P. putida		
UWC1	Hsd$^+$ Hsr$^-$ Rif^r	26
ASR5.2	Activated sludge-derived transconjugants, Km^r 3CB$^+$ encoded by pD10	26
ASR5.10		26
ASR2.8		
Escherichia coli JM107	*thi* Δ(*lac-proAB*) (F′, *traD36 proAB lacI*^{q}ZΔM15) host strain for transformation	49
Strain AS2[a]	Activated-sludge-derived isolate, 3CB$^+$ encoded by pQM300	26
Plasmids		
pD10	Km^r *Sst*I C fragment of pJP4 cloned into pKT231	26
pQM300	3CB$^+$	This study
pNME42	Amp^r *Hin*dIII G fragment of pJP4 cloned into pHG327	This study
pJP4	Hg^r 2,4-D$^+$ 3CB$^+$	12
pHG327	Amp^r	45

[a] CDC group IV C2, not assigned to a genus.

mineral salts medium (41) with 3 mM 3CB alone or with kanamycin, following inoculation with strains harboring pD10. Total bacterial counts in the mixed liquor were estimated by acridine orange staining and epifluorescence microscopy (26).

Laboratory ASU. The laboratory ASU was fed with sterilized wastewater supplemented with artificial sewage (26) with or without 500 mg of 3CB per liter. The ASU was inoculated with activated sludge from a full-scale treatment works (Cynon Valley, South Wales). When 3CB was introduced into the feed, it was added 2 days before inoculation with the pure culture inoculant. The mean residence time in the unit was 24 h, and the sludge wastage was 10% per day. The temperature of the mixed liquor was maintained at 15°C. The performance of the unit was monitored by measuring the biochemical oxygen demand, suspended solids, and mixed-liquor suspended solids by standard methods (2). The numbers of protozoa in the mixed liquor were estimated by using phase-contrast microscopy (26). The ASU was allowed to stabilize for 5 days before inoculation with selected inoculants.

Estimation of 3CB and chloride release. Free chloride produced by the degradation of 3CB in the unit and in batch cultures was estimated by using a Marius Chlor-o-Counter (F. T. Scientific Instruments Ltd., Gloucester, United Kingdom) as described previously (41). In addition, 3CB concentrations in the influent and effluent of the unit were estimated by high-performance liquid chromatography (HPLC) by using an LKB system (model 2152 controller, model 2150 pump, and model 2151 variable-wavelength monitor). Samples for HPLC were centrifuged at 11,600 × *g* in a microcentrifuge (MSE, Crawley, United Kingdom) for 5 min, and the resulting supernatant (1.25 ml) was then diluted to 5 ml with methanol before being used directly for analysis. Separation was carried out on a Lichrosorb RP 18 (10-μm) column (Jones Chromatography, Hengoed, United Kingdom) with a Lichrosorb RP 18 (7-μm) precolumn, with a 75% methanol–25% aqueous phosphoric acid mobile phase. Samples were injected through a rheodyne injection valve with a constant loop size (20 μl), and peaks were detected by measuring A_{283}. Authentic 3CB (BDH) was used as the standard.

Isolation and characterization of plasmid DNA. Plasmid DNA suitable for restriction endonuclease digestion was prepared on a large scale by sucrose gradient centrifugation by the method of Wheatcroft and Williams (48). Small plasmids (<30 kb) were prepared on a large scale by using a modification of the cleared-lysate procedure (9), incorporating a phenol-chloroform purification stage. The method of Holmes and Quigley (20) was used for rapid screening of plasmid DNA in *Escherichia coli* transformants. Restriction endonuclease digestion of plasmid DNA was carried out as recommended by the suppliers (Northumbria Biologicals Ltd., Cramlington, United Kingdom).

DNA cloning and probe preparation. DNA manipulations were done by standard techniques (27). ^{32}P-labeled DNA probes were prepared by nick translation of purified plasmid DNA by using an Amersham N.5000 nick translation kit as recommended by the supplier (Amersham, Buckingham, United Kingdom). [^{32}P]dCTP at 3,000 Ci/mmol (Amersham) was used as the labeling nucleotide and was separated from unincorporated probe by using a Sephadex G-50 column (Pharmacia-LKB Ltd.).

DNA hybridization. Restriction endonuclease-digested plasmid DNA fragments were separated by agarose gel electrophoresis (0.7% [wt/vol] agarose) and were then transferred to a nylon membrane (Hybond-N; Amersham) by Southern blotting (24). DNA hybridizations were carried out as described previously (24) with a final high-stringency wash at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 10 min. Autoradiography was carried out for 18 h by using Fuji RX X-ray film and single intensifying screens (Cronex Lightning-Plus; Du Pont). A DNA probe specific for the catabolic region of plasmid pD10 was constructed by cloning the *Hin*dIII G fragment of pJP4 into the *E. coli* vector plasmid pHG327 (45) to give plasmid pNME52. The *Hin*dIII G fragment of pJP4 contains the *tfdC* gene, encoding chlorocatechol 1,2-dioxygenase, and part of the *tfdD* gene, encoding chloromuconate cycloisomerase (12).

Statistical methods. Statistical analysis of the data was performed as described previously (4).

RESULTS

Survival of *P. putida* UWC1(pD10) in the ASU. The survival in the ASU of *P. putida* UWC1 harboring a recombinant plasmid, pD10, encoding 3CB degradation was described in a previous publication from our laboratory (26). The level of the introduced strain declined from 6.4 × 10^6 to approximately 4.3 × 10^4 CFU/ml over an initial 10-day period without added 3CB. The population remained stable at this level for 14 days and then gradually declined. The population size of degradative bacteria plays an important role in the breakdown of specific compounds in wastewater (7). In the present study, strain UWC1(pD10) was introduced into the ASU mixed liquor at a higher density (3 × 10^8 CFU/ml), with 3CB present in the feed throughout the experiment. Again, the level of the introduced strain declined rapidly over the initial 3 days to 3 × 10^5 CFU/ml and was then maintained for over 10 days with no significant reduction in numbers (Fig. 1). From day 19 onward, the counts on 3CB-kanamycin were significantly different from counts on PSRF plates, indicating that a novel 3CB-degrading population had emerged which

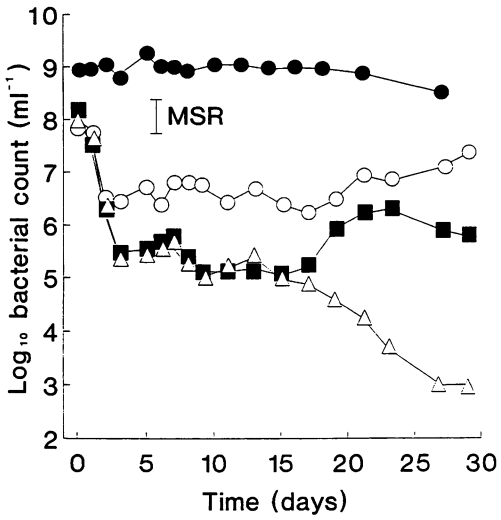

FIG. 1. Survival of *P. putida* UWC1(pD10) in the laboratory-scale ASU. Activated-sludge bacteria were enumerated by using acridine orange direct counts (●) and viable counts on CGY medium (○). Selective media used were *Pseudomonas* agar plus rifampin (△) and 3CB minimal medium plus kanamycin (■). MSR, Minimum significant range.

reached a maximum concentration of 2×10^6 CFU/ml. In addition, from day 19 onward the numbers of the introduced bacterium declined rapidly, falling to $<10^3$ CFU/ml by day 27. In our previous long-term study with the same strain, UWC1(pD10) survived for longer than 8 weeks in the ASU at a population of $>10^3$ CFU/ml (26).

Comparisons of the concentrations of 3CB and free Cl^- in the influent and effluent of the ASU following introduction of UWC1(pD10) indicated that little or no 3CB degradation occurred in the unit before day 15. From day 21 onward, degradation of 3CB was observed and data from HPLC estimations of 3CB showed that 21 to 37% of the available 3CB had been removed. Measurements of free Cl^- indicated that up to 19% of the available 3CB had been mineralized to free Cl^-. The breakdown of 3CB was observed only after the emergence of the natural 3CB-degrading population and so was probably not a direct result of introduction of UWC1(pD10).

Survival of plasmid-free UWC1 in the ASU. The host strain, *P. putida* UWC1 (plasmid-free, $3CB^-$), was introduced into the ASU mixed liquor, and its survival was monitored in separate experiments in which the sewage feed was either supplemented with 3CB (500 mg/liter) or did not contain this compound. In the absence of 3CB, strain UWC1 declined from an initial concentration of 1.8×10^8 CFU/ml to approximately 4.7×10^5 CFU/ml after 6 days (Fig. 2a). The population of the introduced strain then remained stable for the next 10 days. In the presence of 3CB, there was no equivalent stabilization period and a rapid decline in numbers of the introduced strain, to 1×10^3 CFU/ml after 14 days, was observed (Fig. 2b). After 6 days a natural 3CB-degrading population emerged which reached a maximum concentration of 10^7 CFU/ml on day 11.

No breakdown of 3CB was observed before day 10 in the ASU supplemented with this substrate. Breakdown was first observed on day 12 and coincided with detection of the maximum populations of novel 3CB-degrading bacteria. Breakdown increased thereafter to a maximum of 23% of the 3CB available, as determined by HPLC analysis and Cl^- release.

3CB breakdown in batch culture at 15°C. We previously showed that laboratory- and activated-sludge-derived bacteria capable of 3CB degradation in pure culture varied greatly in their ability to mineralize 3CB in supplemented wastewater at 30°C (26). *P. putida* ASR5.10, which showed the

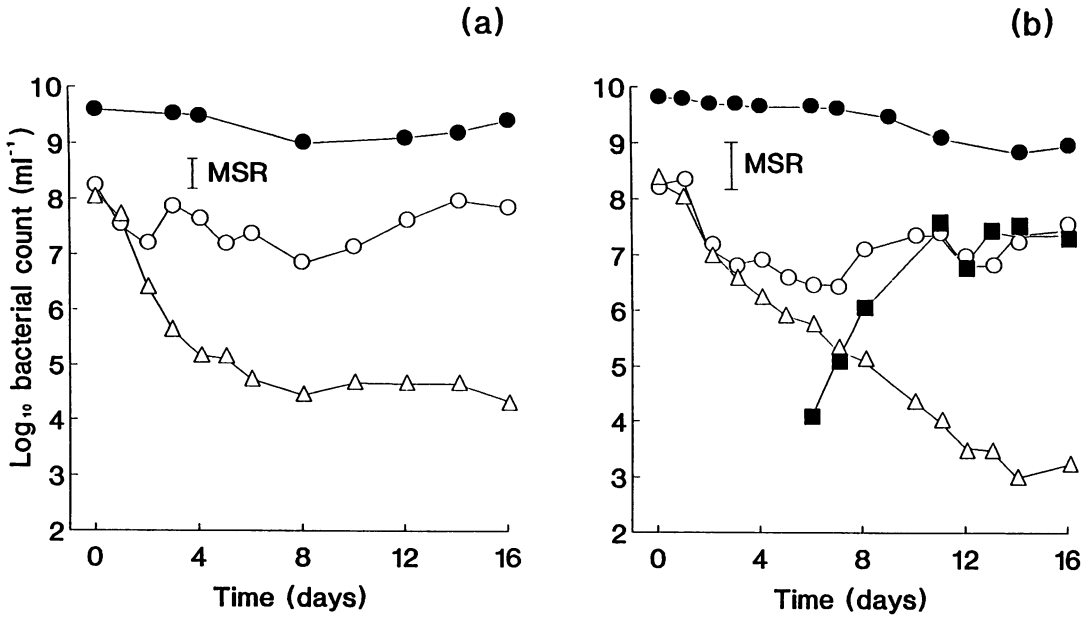

FIG. 2. Survival of *P. putida* UWC1 in the laboratory-scale ASU in the absence (a) and presence (b) of 500 mg of 3CB per liter. Symbols: ●, acridine orange direct count; ○, CGY medium; △, *Pseudomonas* agar plus rifampin; ■, 3CB minimal medium. MSR, Minimum significant range.

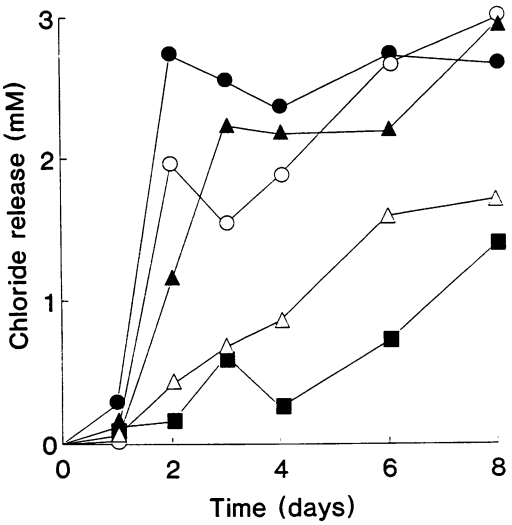

FIG. 3. Rates of chloride release from 3CB by strains in batch culture at 15°C. Strains shown are *P. putida* UWC1(pD10) (■), activated-sludge isolate AS2 (●) and activated-sludge-derived transconjugant strains *P. putida* ASR5.2 (○), *P. putida* ASR2.8, (▲), and *P. putida* ASR5.10 (△).

highest rate of Cl^- release at 30°C, did not enhance 3CB breakdown when inoculated into the ASU (data not shown). Since the routine operational temperature of the ASU was 15°C, further batch culture experiments were carried out to determine the rate at which activated-sludge-derived bacteria, containing plasmid pD10, and strain AS2 degraded 3CB in supplemented wastewater at 15°C. Strain UWC1(pD10) degraded 3CB very slowly at this temperature, and *P. putida* ASR5.10, which showed the highest rate of Cl^- release at 30°C, also proved relatively ineffective (Fig. 3). The highest rates of Cl^- release at 15°C were observed with the natural isolate strain AS2 and the activated-sludge-derived transconjugant bacteria *P. putida* ASR2.8 and ASR5.2. Strains AS2 and *P. putida* ASR2.8 were selected for further study.

Survival of strain AS2 and *P. putida* ASR2.8 in the ASU. To facilitate detection of the introduced strains, spontaneous rifampin-resistant mutants of strains AS2 and *P. putida* ASR2.8 were isolated and used in ASU experiments. Strain AS2 (Rif^r) was introduced into the ASU at an initial concentration of 2×10^8 CFU/ml; it did not show an immediate rapid population loss, but maintained a relatively high level in the aeration chamber over the 16-day incubation period. There was a significant fall in numbers on day 8, but following this the population stabilized at approximately 3×10^6 CFU/ml (Fig. 4a). Under the same conditions, *P. putida* UWC1, with or without plasmid pD10, declined to $<10^5$ CFU/ml after the same period (Fig. 1 and 2b, respectively). *P. putida* ASR2.8 (Rif^r, carrying plasmid pD10) was introduced into the unit at 8×10^7 CFU/ml, and over the initial 7-day period the numbers fell to approximately 10^6 CFU/ml (Fig. 4b) but then remained stable for the next 10 days. Both strain AS2 and *P. putida* ASR2.8 were retained in the ASU mixed liquor at higher populations than were previously observed for the other introduced bacteria.

Chloride release, indicative of 3CB mineralization in the ASU, was detected 3 days after the introduction of strain AS2 into the mixed liquor (Table 2). HPLC estimations of 3CB concentrations in the influent and effluent indicated that 14 to 27% of the available 3CB was degraded in the unit from days 4 to 16. Chloride release and 3CB degradation were also detected 4 days after the introduction of *P. putida* ASR2.8 into the ASU and were commensurate with the breakdown of 12 to 19% of the 3CB available from days 5 to 17. In contrast to previous experiments conducted with other introduced strains, no natural 3CB-degrading populations emerged (bacterial counts on complex media with rifampin

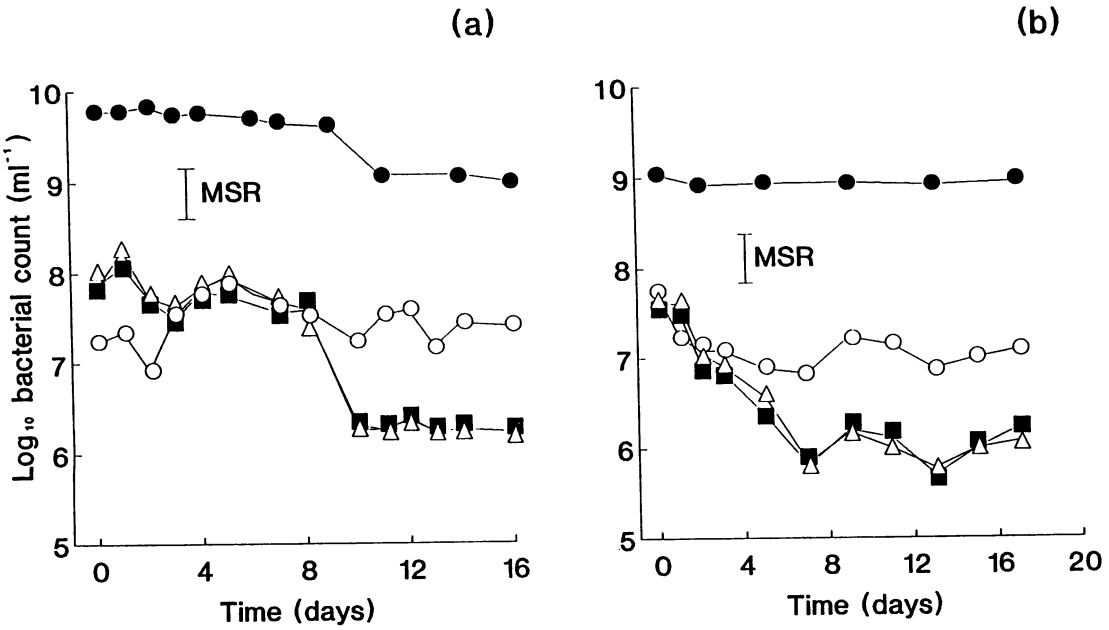

FIG. 4. Survival of strain AS2 (a) and *P. putida* ASR2.8 (b) in the laboratory-scale ASU. Symbols: ●, acridine orange direct count; ○, CGY medium; △, L agar plus rifampin; ■, 3CB minimal medium with or without kanamycin (kanamycin present in panel b). MSR, Minimum significant range.

TABLE 2. 3CB breakdown in the ASU following inoculation with strain AS2 and *P. putida* ASR2.8

Day	% Cl^- release[a] in ASU effluent after inoculation with:	
	AS2	*P. putida* ASR2.8
0	0	0
1	2	4
3	9.5	6
5	19.5	21
7	21	19
9	18.5	19
11	20.5	18
13	28	16
15	20.5	19
17	26	16

[a] Total (100%) degradation of 3CB would yield 3.2 mM Cl^-.

were not significantly different from counts on 3CB medium).

Performance of the ASU. The performance of the ASU as a waste treatment unit was assessed in all the experiments, and the unit consistently produced a good-quality effluent with substantial reductions in biochemical oxygen demand and suspended solids concentrations, similar to those previously observed (26). The mean mixed-liquor suspended solids concentrations were between 1,000 and 1,800 mg/liter, and the settling characteristics of the sludge were good (sludge volume index below 200). Total levels of bacteria remained high (about 10^9 to 10^{10}/ml [Fig. 1, 2, and 4]), and numbers of viable heterotrophs varied little once the inoculants had stabilized. In all the experiments large populations of ciliated protozoa were observed, which were dominated by peritrichs and crawling ciliates commonly associated with well-run large-scale units (32). No effects on protozoal numbers were observed after the bacterial inoculations.

Characterization of strain AS2. Strain AS2, a gram-negative, oxidase-positive, short rod-shaped bacterium, was originally isolated from the ASU run at 15°C in the presence of 1,000 mg of 3CB per liter (26). It produced a characteristic mucoid mass when grown in shaken liquid culture, which readily settled when shaking stopped, and it produced mucoid colonies on solid media. Other characteristics of this strain were as follows: negative results for nitrate reduction, indole production, arginine hydrolase, and gelatin liquefaction, and positive assimilation of gluconate, caprate, adipate, malate and phenylacetate. Detailed biochemical tests on strain AS2 (NCIMB Ltd., Aberdeen, Scotland) did not result in a clear classification, but the organism was identified as a member of CDC group IV C2, which has not yet been assigned to a genus. Because strain AS2 was isolated from the ASU in an experiment in which the unit was originally inoculated with strain UWC1(pD10), it was important to determine whether the ability of strain AS2 to grow on 3CB involved genes resident on or derived from the recombinant plasmid. Strain AS2 contained at least one large plasmid (data not shown) and, when grown in L broth in the presence of mitomycin C (1 μg/ml), readily lost the ability to grow with 3CB (>90% loss after a 48-h incubation). In addition, strain AS2 transferred the ability to grow on 3CB to a plasmid-free recipient, strain UWC1, in a plate filter mating. The resulting transconjugants carried a single large plasmid (data not shown), which was designated pQM300.

Hybridization studies with pJP4-derived catabolic genes. Plasmid DNA from strains AS2 and UWC1(pQM300) was digested with *Hin*dIII and then hybridized with ^{32}P-labeled pNME52 or pJP4 under conditions of high stringency. No hybridization between the probe pNME52 and plasmid DNA from strain AS2 or UWC1(pQM300) was observed (Fig. 5). Control DNA (purified plasmid pJP4 cut with *Hin*dIII) hybridized strongly with this probe. However, when pJP4 was used as the probe DNA, hybridization was observed with fragments of DNA isolated from both strain AS2 (four fragments showing homology [Fig. 5]) and strain UWC1 (pQM300) (two fragments showing homology [Fig. 5]). Although plasmid pQM300 showed homology with pJP4 under conditions of high stringency, the lack of hybridization observed with plasmid pNME52 indicated that the genes responsible for 3CB catabolism on pQM300 were not homologous to the *tfdC* and *tfdD* genes resident on pD10. The hybridization observed with the broad-host-range conjugal plasmid pJP4 may have resulted from homology with other functions, for example replication, transfer, or other catabolic regions present on the plasmid.

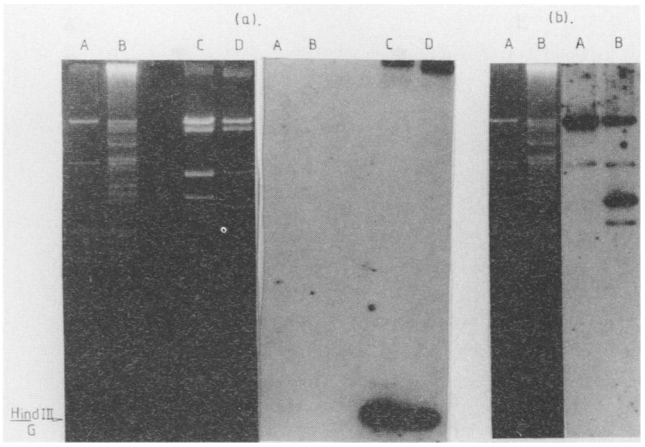

FIG. 5. Agarose gel electrophoresis and autoradiograms of *Hin*dIII digests of plasmid DNA hybridized with a pNME52 DNA probe (a) and a pJP4 DNA probe (b). Lanes: A, pQM300 (from *P. putida* UWC1); B, plasmid DNA from strain AS2; C, pJP4Δ1 (deleted derivative of pJP4 retaining the *Hin*dIII G fragment); D, pJP4.

DISCUSSION

In the absence of 3CB, the survival of plasmid-free *P. putida* UWC1 followed a pattern similar to that observed previously with the same host bacterium harboring the recombinant plasmid pD10 (26), indicating that the presence of plasmid pD10 did not have a serious detrimental effect on the persistence of the host strain. In both cases, after an initial rapid decline in numbers of the introduced organism, the UWC1 population stabilized at between 10^4 and 10^5 CFU/ml. The presence of 3CB in the ASU feed had a detrimental effect on the survival of strain UWC1, with or without plasmid pD10. This may have resulted from a direct toxic effect of 3CB or a metabolite derived from the substrate (strain UWC1 can convert 3CB to the corresponding chlorocatechol by using chromosomally encoded enzymes). Alternatively, the large increase in numbers of natural 3CB-degrading bacteria may have deleteriously affected the growth or survival of UWC1 in the ASU ecosystem. This latter explanation seems the most likely because a rapid decline of the introduced strains UWC1 and UWC1(pD10)

coincided with the emergence of a natural (enriched) 3CB-degrading population. Also, in our previous study (26), a population of UWC1(pD10) inoculated into the ASU in the presence of 1,000 mg of 3CB per liter did not drop below 3×10^5 CFU/ml when other 3CB-degrading strains were absent. Several studies have shown that competition with the autochthonous microflora can adversely affect the survival of introduced strains in experimental microcosms (34, 38, 47).

The results described in this paper illustrate the importance of selecting bacterial inoculants with appropriate physiological characteristics for survival and in situ activity in target ecosystems. The laboratory strain UWC1(pD10) did not enhance 3CB breakdown in the ASU, despite introduction at a high population density. This was not the result of instability of the catabolic phenotype encoded by plasmid pD10, nor was it due to loss of viability following introduction into the ASU. In this and the previous study (26), introduced organisms persisted in the ASU for long periods (many weeks) and the recombinant plasmid pD10 was retained in a high proportion (>90%) of the introduced populations, indicating both structural and segregational stability.

We have proposed that the inability of strain UWC1(pD10) to degrade 3CB in supplemented wastewater may have been due to the availability of alternative substrates (26). Industrial wastewaters usually contain mixtures of chemicals, some of which are available as nutrient sources (carbon, nitrogen, etc.), both readily utilizable and poor substrates, and others which can inhibit bacterial growth. Swindoll et al. (46) suggested that the mineralization of xenobiotic substrates in a groundwater aquifer by microorganisms was repressed in the presence of an alternative, more easily degradable carbon source. Further experimental support for this type of catabolite repression was reported by Schmidt and Alexander (40), who observed that alternative substrates, such as acetate and glucose, added at high concentrations, repressed catabolism of relatively low concentrations of phenol and aniline by *Pseudomonas* spp. and *Salmonella typhimurium*. Results described here from batch culture experiments indicated that temperature can also affect the ability of an inoculant to function in situ. Thus, strains AS2 and *P. putida* ASR2.8 degraded 3CB in the ASU, but *P. putida* ASR5.10, which mineralized 3CB rapidly in batch culture at 30°C but not at 15°C, did not.

Dwyer et al. (13) reported that although genetically manipulated derivatives of *Pseudomonas* sp. strain B13 survived well in laboratory activated-sludge microcosms, their performance in this complex ecosystem was poor compared with their biodegradative activity towards mixed substrates in pure culture. In this paper we have described the selection of 3CB-degrading bacteria from the activated sludge, both by conventional enrichment (e.g., strain AS2) and by genetic manipulation (e.g., *P. putida* ASR2.8 and ASR5.2), which were capable of mineralizing the substrate at high rates in wastewater. Hybridization studies with pJP4-derived catabolic genes showed that the ability of strain AS2 to catabolize 3CB was not the result of acquisition of plasmid pD10 but that this strain harbors a novel 3CB catabolic plasmid. Bioremediation of a 3CB-contaminated activated sludge was possible by using inoculants formulated with these strains, presumably because they were adapted to that particular ecosystem. However, we can only speculate on the specific characteristics of the selected strains which resulted in the observed enhancement of 3CB biodegradation. Alexander and his colleagues have suggested a number of plausible reasons that may explain the failure of laboratory-selected microbial inoculants to enhance biodegradation (1, 17, 40). In this context, Kobayashi (23) and Alexander (1) discussed the importance of choosing well-adapted organisms in the formulation of effective inoculants for wastewater treatment processes.

The factors that are most frequently suggested in the literature to influence the growth and survival of organisms introduced artificially to natural habitats (6) are predation, competition, resistance to starvation, motility, nutrient (substrate and cofactor) concentration and availability, presence of growth inhibitors, and physical factors such as O_2 concentrations, pH, and temperature. These factors may act either individually or synergistically to exert their effect. The two strains which were observed to degrade 3CB after inoculation into the ASU, *P. putida* ASR2.8(pD10) and strain AS2, were both selected for their ability to mineralize 3CB in batch culture on supplemented wastewater at 15°C. Significantly, these strains maintained stable populations ($>10^6$ CFU/ml) in the ASU after inoculation. The characteristic flocculation of strain AS2 may have been an important factor in the maintenance of a stable population in the ASU (Fig. 4a). It was noticeable that immediately following inoculation there was no rapid decline in the numbers of strain AS2, in contrast to the other inoculants tested. The ability of an introduced organism to colonize successfully and persist in a new habitat will clearly affect its ability to enhance biodegradation or its other beneficial functions in situ. In addition, the retention of inoculated organisms in continuous-flow reactors is important for reasons of process efficiency and cost; hence the interest in the use of biofilms and immobilized enzyme systems for wastewater treatment (29). The decline in the AS2 population on day 8 was accompanied by reductions in both total bacterial numbers and heterotrophic plate counts, suggesting some perturbation to the functioning of the ASU. However, 3CB breakdown appeared to be unaffected, and the AS2 and heterotrophic populations stabilized thereafter. Such dramatic population declines have not been observed in subsequent experiments in which strain AS2 has been inoculated into a functional ASU.

The results presented, both in this paper and previously (26), indicated that inoculation of the ASU with large numbers of bacteria harboring a recombinant plasmid did not adversely affect the functioning of the unit or the protozoa present. Mean numbers of protozoa and the population structure were similar to those found in full-scale treatment plants (10, 11, 32). As the protozoa in activated-sludge tanks feed mainly on bacteria, the inoculation of the ASU with genetically engineered bacteria did not seriously affect the higher trophic levels present.

During this study we used selective plate counts exclusively to enumerate bacteria introduced into the activated-sludge ecosystem. Fulthorpe and Wyndham (15) recently showed that viable counts were an unreliable method of determining the survival of a 3CB-degrading catabolic genotype in lake microcosms. In their study, discrepancies between viable counts on 3CB agar and most-probable-number–DNA hybridization estimations were attributed to catabolic instability in the original inoculant and to transfer of the conjugal 3CB-catabolic plasmid, pBR60, to indigenous hosts. We have used selective media, which allowed us to monitor both the host bacterium and the plasmid-encoded phenotype, and obtained no evidence for catabolic instability in inoculants harboring plasmid pD10 nor for transfer of the recombinant plasmid to indigenous bacteria.

A number of genetic and immunological methods have been developed or adapted to allow the detection of specific

bacteria or gene sequences in environmental samples (8, 19, 28, 36). The development of methods allowing the extraction of total microbial genomic DNA from environmental samples (8, 30), coupled with the amplification of specific DNA sequences by using the polymerase chain reaction (8, 43), allows the sensitive detection of specific organisms or gene sequences without requiring growth or colony formation on laboratory media. The nucleotide sequence of the *tfdC* gene encoding the chlorocatechol 1,2-dioxygenase resident on plasmid pD10 has been determined (16). In addition, methods for the extraction of DNA from sewage samples have been developed (25a), and the sensitive detection of bacteria harboring plasmid pD10 should be possible, irrespective of their ability to grow on laboratory media. The plasmid probe pNME52 described in this paper has also been used to identify bacteria harboring pD10 in mixed liquor by colony hybridization (27). Steffan et al. (44) have suggested that multiple methodological approaches should be used to achieve accurate and sensitive monitoring of specific bacteria in environmental samples.

That significant levels of 3CB degradation were observed in situ, after inoculations of ASUs with selected recombinant and natural 3CB-utilizing bacteria in the presence of alternative substrates, predatory protozoa, and a competitive indigenous microflora, and at relatively low temperatures, indicated that the selection strategy we have used was valid. Improvements in the bioremediation process may be achieved in a number of ways. For example, further selection of the existing 3CB-degrading strains or isolation of better alternative strains, capable of higher rates of 3CB catabolism and exhibiting a lower K_s for 3CB, in the ASU mixed liquor could facilitate the formulation of improved inoculants. Alternatively, rates of biodegradation may be increased through optimization of the process operational parameters. For the ASU this might involve raising the temperature of the mixed liquor, altering the residence time, or increasing the sludge return rate.

The development and testing of model systems (microcosms) which accurately mimic real ecosystems are important in determining the fate and efficiency of microbial inoculants. The laboratory-scale ASU described here and in our previous paper (26) has provided a contained microcosm mimicking the full-scale aerobic treatment process and has facilitated the isolation of catabolic genetically engineered microorganisms and natural 3CB degraders and the evaluation of inoculants for bioremediation of contaminated activated sludge.

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.

We thank S. Rees for technical help and B. Evans for the HPLC analysis.

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