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Vol. 9(1), pp. 17-25, 7 January, 2015
DOI: 10.5897/AJMR2014.7190
Article Number: B3A0F6250000
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

*Full Length Research Paper*

# Microbial degradation of 4-chloroaniline by a bacterial consortium

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Received 14 October, 2014; Accepted 15 November, 2014

**In this study, we have isolated four different species from effluent contaminated soil using a mixture of aniline and 4-chloroaniline (4CA) as principal carbon sources. The four species were identified as *Pseudomonas stutzeri* A, *Comamonas testosterone* B, *Pseudomonas putida* C and *Stenotrophomonas maltophilia* D. Growth studies on aniline and 4CA as single and mixed substrates demonstrated that the bacteria preferred to grow on and utilize aniline rather than 4CA. However, despite 100% disappearance of the parent substrates, the degradation of 4CA was always characterized by incomplete dechlorination and 4-chlorocatechol accumulation. HPLC-UV analysis showed that 4-chlorocatechol was further degraded via an ortho-cleavage pathway by the bacterial consortium. This hypothesis was supported by the results from enzyme assays of the crude cell extract of the consortium revealing catechol 1,2-dioxygenase activity which converted catechol and 4-chlorocatechol to cis,cis-muconic acid and 3-chloro-cis,cis-muconic acid, respectively. However, the enzyme had a much higher conversion rate for catechol than for 4-chlorocatechol, indicating preference for non-chlorinated substrates. Members of the bacterial consortium were also characterized individually. All isolates were able to assimilate aniline. *P. putida* C was able to grow on 4CA solely, while *S. maltophilia* D was able to grow on 4-chlorocatechol. These results suggest that the degradation of 4CA in the presence of aniline by the bacterial consortium was a result of interspecies interactions.**

**Key words:** Chloroaniline, *Pseudomonas*, *Comamonas*, HPLC, catechol.

## INTRODUCTION

4-Chloroaniline is one of the chlorinated aromatic amines that have been extensively used in the industrial production of dyes, cosmetics, pharmaceutical products and herbicides (Kearney and Kaufmann, 1975; Latorre et al., 1984). It has been registered in the high production volume chemical program of the Organization for Economic Co-operation and Development (OECD, 1997). Not only distributed through the industrial production, 4-chloroaniline is also one of the primary intermediates pre-dominantly generated by microbial transformation of phenylurea, acylanilide and phenylcarbamate herbicides (Zeyer and Kearney, 1982; Haggblom, 1992). As a consequence of its intensive applications, it has been ubiquitous and accumulated in the environment including industrial

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effluent, sludge as well as agricultural soil. Due to its toxicity and recalcitrant properties, it has been considered as one of the important environmental pollutants (EEC, 1976; Federal Register, 1979). To dissimilate the toxic compound, bioremediation can be applied as one of the remediation technologies in which a decontamination process depends on the microbial biodegradability. Therefore, many efforts have been undertaken to isolate bacteria capable of biodegradation of chloroanilines (Surovtseva et al., 1985; Loidl et al., 1990). Although some microorganisms have been reported to have biodegradability towards chloroanilines, their limitation is that they are unable to grow on chloroanilines as sole sources of carbon and nitrogen (Helm and Reber, 1979; Reber et al., 1979; You and Bartha, 1982; Radianingtyas et al., 2003). Only recently, 3-chloroaniline-degrading *Comamonas testosteroni* strain I2gfp capable of utilizing 3-chloroaniline as a sole source of carbon and nitrogen was isolated (Boon et al., 2000). Nonetheless, under certain growth conditions, a few microorganisms have been reported to be able to grow on 4-chloroaniline as a sole carbon and nitrogen source. For instance, previous studies stated that the bacterial isolations were successful only after they were cocul-tivated or induced in the presence of aniline or another cosubstrate, for example, glucose, for some periods of time (Zeyer and Kearney, 1982; Radianingtyas et al., 2003; Travkin et al., 2003). In the current study, we used a defined bacterial consortium, reconstructed from strains isolated from a putatively herbicide-contaminated site, to investigate 4-chloroaniline (4CA) metabolism in the presence of aniline. Each member of the consortium was identified and characterized in order to determine their role in the degradation process.

## MATERIALS AND METHODS

### Media and chemicals

3, 4-Dichloroaniline and 4-chloroaniline (99% purity; Chem Service) were dissolved in high-quality methanol (Fisher Scientific) before use. Aniline (Merck, Germany), 2-chloroaniline and 3-chloroaniline (99% purity; Chem Service) were used as liquid. A minimal medium (MM; pH 7.0) was used according to Zeyer et al. (1985). The media were solidified with 15 $gL^{-1}$ of high-quality agar (Scharlau Microbiology, Spain) for cell growth on plate.

### Enrichment, isolation and identification

Soil samples were used to enrich a percolating soil column containing 200 ml basal medium: 0.45 g $KH_2PO_4$, 1.2 g $Na_2HPO_4$, 0.1 g $MgSO_4.7H_2O$, 0.05 g $CaSO_4.2H_2O$ and 3.35 mg trace elements per litre (Zeyer and Kearney, 1982). The trace elements solution was composed of 1 mg each of $FeSO_4.7H_2O$ and $MnSO_4.H_2O$; 0.25 mg each of $(NH_4)6Mo_7O_{24}.4H_2O$, $Na_2B4O_7.10H_2O$, $Co(NO_3)2.6H_2O$, $CuCl_2.2H_2O$ and $ZnCl_2$; plus 0.1 mg $NH_4VO_3$. The pH was adjusted to 7.0 with 5 M NaOH. The medium was then steam-sterilized and cooled prior to addition of filter-sterilized aniline and 4CA to avoid thermal destruction of the substrates. Soil samples (2 g) were placed onto a layer of glass beads and glass wool in the neck of the column. Samples of the liquid were plated out weekly onto solidified basal medium (2%, w/v, Lab M agar) containing 4CA plus aniline and incubated at 30°C. Morphologically different colonies were selected and sub-cultured onto solid basal medium containing the same substrates. The purity of the cultures was checked by plating them onto nutrient agar medium. The single isolates obtained were recombined into a bacterial consortium by making individual bacterial suspensions with equal OD600 and 200 ml of each suspension was added to 50 ml fresh basal medium in a 250 ml flask for subsequent experiments. The isolates chosen for further research were identified according to their phenotypic and genotypic characters. Preliminary characterization was based on colony morphologies on tryptic soy agar and Gram staining. Further identification was done using the API 20NE test, fatty acid methyl ester (FAME) analysis and 16S rRNA gene sequencing.

### API 20NE test

Bacterial suspensions were prepared using isolates on nutrient agar. Colonies were picked up and suspended in sterile 0.85% NaCl medium to get turbidity equivalent to 0.5 McFarland. The suspension was then added to the tubes according to the manufacturer's instruction. Reading of reaction within each test was taken after 24 h incubation at 30°C. A second reading for substrate assimilation test was taken after 24 h incubation at 30°C. The results were then interpreted for bacterial identification by coding the reaction patterns into 7-digit numerical profiles according to the manufacturer's instruction and isolates were identified using the Analytical Profile Index (1990 edition).

### 16S rRNA gene sequencing

Genomic DNA from individual bacterial strains was extracted according to Pitcher et al. (1989). The 16S rDNA was selectively amplified from genomic DNA by using PCR with oligonucleotide universal primers Eubac 27F and Eubac 1492R (DeLong et al., 1993). PCR amplification was undertaken with a DNA Thermal Cycler model 9600 (Perkin Elmer Cetus) under the following conditions: 100-200 ng template DNA, 10 ml 10*0* reaction buffer (100 mM Tris/HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3 at 20°C), 2.5 U Taq DNA polymerase (Boehringer Mannheim), 1 mM upstream primer, 1 mM downstream primer, 200 mM of each dNTP (Promega) and PCR $H_2O$ (Sigma-Aldrich) combined in a total volume of 100 ml. The tubes were incubated at 95°C for 5 min and then subjected to the following thermal cycling programme: denaturation at 95°C for 45 s, primer annealing at 55°C for 45 s, and chain extension at 72°C for 2 min with an additional extension time of 10 min on the final cycle, for a total of 40 cycles. The amplified DNA was purified using Qiaquick PCR Purification Kit (Qiagen), adjusted to 200 ng $\mu l^{-1}$, for sequencing using Eubac 27F, Eubac 1492R (DeLong et al., 1993) and Eubac 357F (Colquhoun et al., 1998). Fragments of 16S rDNA (1073-1430 bp) were compared with the most similar sequences in the GeneBank and EMBL nucleotide sequence databases based on percentage similarities.

### Degradation experiment

Experiments were done in 250 ml flasks at 30°C with constant shaking. Growth was monitored spectrophotometrically by measuring culture turbidity at 600 nm. Depletion of target compounds (as measured by HPLC) and the liberation of $Cl_2$ ion were used as indicators of substrate utilization. The growth kinetics of the bacterial consortium when grown on aniline or on 4CA over a range of initial substrate concentrations (0.2-3.0 mM) was determined.

**Table 1.** Identification.

| Strain | API 20NE | | FAME | | Partial 16S rDNA | |
|---|---|---|---|---|---|---|
| | Most similar species* | id † (%) | Most similar species | Index‡ | Most similar species (accession no.) | Similarity (bp overlap) (%) |
| A | *Agrobacterium radiobacter* | 99.9 | *Flavobacterium balustinum* | 0.028 | *Chryseobacterium indologenes (M58773)* | 95.5 (1309) |
| B | Unidentified | - | *Comamonas testosteroni* | 0.546 | *Comamonas testosteroni* (AB00796) | 99?7 (1096) |
| C | Unidentified | - | *Comamonas testosteroni* | 0.81 | *Pseudomonas agarici* (D84005) | 98?2 (1405) |
| D | Xanthomonas maltophilia | 99.2 | Not determined | - | *Pseudomonas hibisciola* (AB021405) | 99.6 (1069) |
| | | | | | *Stenotrophomonas maltophilia (X95923)* | 99.5 (1068) |

API 20NE (Analytical profile index 1990 edition). †Percentage id is the estimate of how closely the profile of the unknown taxon matches all other taxa in the database. ‡Index values areobtained from a covariance matrix showing relatedness of one fatty acid to another between an unknown microbial sample and a library of identified species. The lower the index, the higher the probability of a match.

The effect of one substrate on the utilization of the other by the bacterial consortium was also analyzed by monitoring growth over a range of aniline and 4CA concentrations. The specific growth rates of the bacterial consortium at one fixed aniline concentration and various 4CA concentrations (0.2 mM aniline with 0.2, 0.5, 1 and 3 mM 4CA) were determined, and the same procedure was also applied to analyze the reciprocal effects of 4CA on aniline utilization.

### Enzyme activity

Cell-free extracts of the bacterial consortium grown on a mixture of 1 mM aniline and 1 mM 4CA were prepared according to Dorn and Knackmuss (1978). Freshly harvested bacteria suspended in 20 mM Tris/HCl buffer pH 8.0 containing 1 mM dithiothreitol were disrupted by passage at least wice through an Aminco French pressure cell at 6.9 MPa. Cell debris was removed by centrifugation at 15,000 g for 40 min and the supernatant was decanted and used as the cell free extract. Enzyme activities were measured spectrophotometrically according to Blasco et al. (1995). Catechol 1, 2-dioxygenase activity was measured by following the formation of cis,cis-muconate at 260 nm and catechol 2,3- dioxygenase activity by the formation of hydroxymuconic semialdehyde at 378 nm. Reaction mixtures initially contained 33 mM Tris pH 8.0, 2 mM EDTA, and an appropriate amount of enzyme. After 5 min incubation at 30°C, the reaction was started by the addition of catechol or 4-chlorocatechol to a final concentration of 0.2 mM. Protein was determined by the method of Bradford (1976) using the Coomassie Blue Protein Assay Reagent Kit from Pierce with bovine serum albumin used as the standard (100-1500 mg $ml^{-1}$). The solution was mixed and the A595 measured on a Lambda 15 UV spectrophotometer.

### Analytical methods

HPLC analysis was done with a Perkin Elmer instrument equipped with a UV detector at a wavelength of 254 nm. Isocratic separations were done on a reverse-phase Radial-PAK C18 column (2 mm diameter; particle size 5 mm). A water/methanol mixture (30: 70, v/v) was supplied at a flow rate of 1 ml $min^{-1}$ as the mobile phase. The separation of anilines required a 10% (v/v) addition of an aqueous solution of $NH_4H_2PO_4$ (0.1 M, pH 3.0) to the mobile phase. Qualitative and quantitative data were obtained by comparing the peak area of unknowns with those of standards of known concentrations. Aniline, 4CA, catechol, 4-chlorocatechol, muconic acid and 3-oxoadipic acid were purchased from Sigma-Aldrich. Free chloride concentration in the culture supernatant was determined spectrophotometrically using the method of Bergman and Sanik (1957).

## RESULTS

### Isolation, identification and biochemical characterization

Growth of the bacterial culture was observed after 5 days, during which time the colour of the liquid percolating through the column changed from colorless to dark yellow. Samples were plated out each week onto basal medium containing 4CA or aniline and incubated at 30°C for 7-10 days. After subsequent purification and sub culturing, four distinct isolates, designated as A, B, C and D were obtained and recombined into a bacterial consortium. All members of the bacterial consortium were Gram negative, rod-shaped, motile, non-sporulating and catalase and oxidase-positive. All isolates produced entire, smooth, glossy, convex and opaque colonies on tryptic soy agar plates. However, the consortium members were readily distinguished on the basis of colony charac-teristics: those of isolate A were orange and circular with diameter 2-6 mm; those of isolate B were off-white and circular with diameter 1-3 mm; those of isolate C were white and circular with diameter 3-7 mm; and those of isolate D were bright yellow, opaque and circular with diameter 2-4 mm. The results of further identification using the API 20NE test system, followed by FAME profiling and partial 16S rDNA sequencing analyses, are presented in Table 1. Integrated information of the phenotypic and genotypic characters of the four isolates led to them being identified as Chryseobacterium (previously Flavobacterium: Vandamme et al., 1994) *Pseudomonas stutzeri* A, *Comamonas testosterone* B, *Pseudomonas putida* C and *Stenotrophomonas maltophilia* D.

### Metabolic characterization

In batch cultures, the bacterial consortium was able to

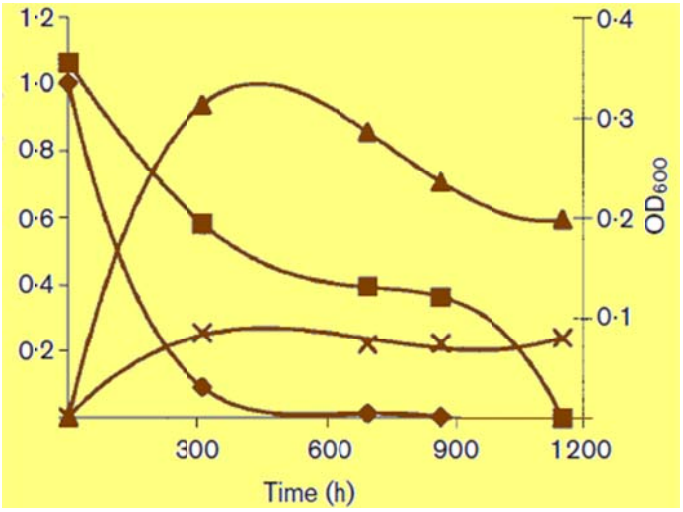


**Figure 1.** Growth ($OD_{600}$) of the bacterial consortium (Δ) and 4CA utilization as shown by dechlorination level ($Cl^-$ in supernatant, x) on an aniline/4CA mixture. The initial concentrations of 4CA (□) and analine (◊) were each 1 mM. Symbols represent means of experimental values in duplicate and lines were fitted to polynomial curves of the best order ($3^{rd}$-$5^{th}$) using Microsoft Excel 2002.

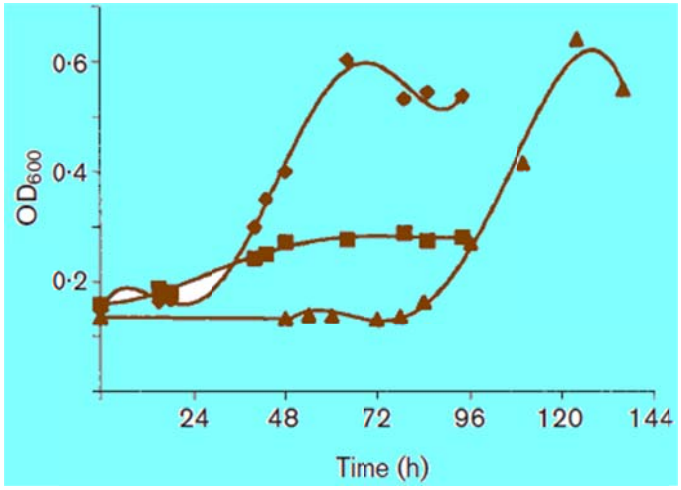


**Figure 2.** Growth profile of the bacterial consortium on 1 mM aniline (◊), 1 mM 4CA (□) and a mixture of 1 mM aniline and 1 mM 4CA (Δ).Symbols represent means of experimental values in duplicate and lines were fitted to polynomial curves of the best order ($3^{rd}$-$5^{th}$) using Microsoft Excel 2002.

degrade 4CA in the presence of aniline (Figure 1). Aniline was almost completely removed after 312 h incubation, while 4CA remained detectable in the culture. However, despite 100% disappearance of 4CA at the end of observation period (1152 h), dechlorination stopped at a chloride concentration of 0.25 mM (Figure 1). During the first 168 h of incubation a greenish-yellow colouration of the culture filtrate was observed. After 240 h, the colour of the culture turned dark brown and accumulation of 4-chlorocatechol in the culture supernatant was detectable by HPLC. In order to evaluate the effect of each substrate on bacterial growth, the consortium was grown on aniline or 4CA individually, or as a mixture. When the consortium was challenged with a mixture of 1 mM aniline and 1 mM 4CA, it showed a longer lag phase (72 h as compared to 18 h) and a higher specific growth rate (0.034±0.004 h21) than when each substrate was supplied alone (0.027±0.003 h21 for aniline and 0.014±0.006 h21 for 4CA) (Figure 2). When aniline and 4CA were supplied individually, the bacterial consortium had higher mmax and Ks for aniline (0.037±0.006 h21 and 0.074±0.006 g l21 respectively) than for 4CA (0.020±0.006 h21 and 0.022±0.005 g l21 respectively), that is, a higher specific growth rate on aniline but a higher affinity for 4CA. In subsequent experiments, the growth of the bacterial consortium was examined on aniline/4CA mixtures following a matrix combination (see Methods: degradation assays). The effect of 4CA concentration on the growth of the bacterial consortium at defined concentrations of aniline is shown in Figure 3a. 4CA at 3 mM inhibited bacterial growth at any concentration of aniline. On the other hand, the presence of aniline did not inhibit bacterial growth on 4CA, regardless of the concentration of 4CA (Figure 3b). In order to investigate the reason for the incomplete dechlorination, the degradation pathway of 4CA by the bacterial consortium was examined. Analyses of the culture filtrate by HPLC-UV revealed peaks that were similar to 4-chlorocatechol (3.92 min) and 3-oxoadipic acid (2.32 min) reference standards. The HPLC-UV analysis also showed peaks of unknown metabolites which appeared at 2.5 and 5.52 min. Further investigation of the 4CA degradation pathway was made by determining catechol 1, 2-dioxy-genase and catechol 2,3-dioxygenase activities of the consortium grown on a mixture of 1 mM aniline and 1 mM 4CA. The crude bacterial extracts transformed catechol and 4-chlorocatechol to cis, cis-muconic acid and chloro-cis, cis-muconic acid respectively, indicating the activity of catechol 1, 2-dioxygenase, but not to 2-hydroxymuconic semialdehyde, the intermediate product from catechol 2, 3-dioxygenase activity. The specific activity of catechol 1, 2-dioxygenase in crude extracts of the bacterial consortium was higher against catechol than that against 4-chlorocatechol (Table 2).

## Characterization of consortium

To assess the possible role of each member of the bacterial consortium in 4CA degradation, their ability to grow as mono species cultures on 4CA in the presence of aniline was investigated. All the strains grew on mixed 1 mM aniline and 1 mM 4CA with specific growth rates ranging from 0.023 to 0.055 h21; *Pseudomonas putida* C had the highest specific growth rate. Similarly, all strains had lag phases between 48 and 72 h before exponential growth commenced. Whereas all four strains could grow

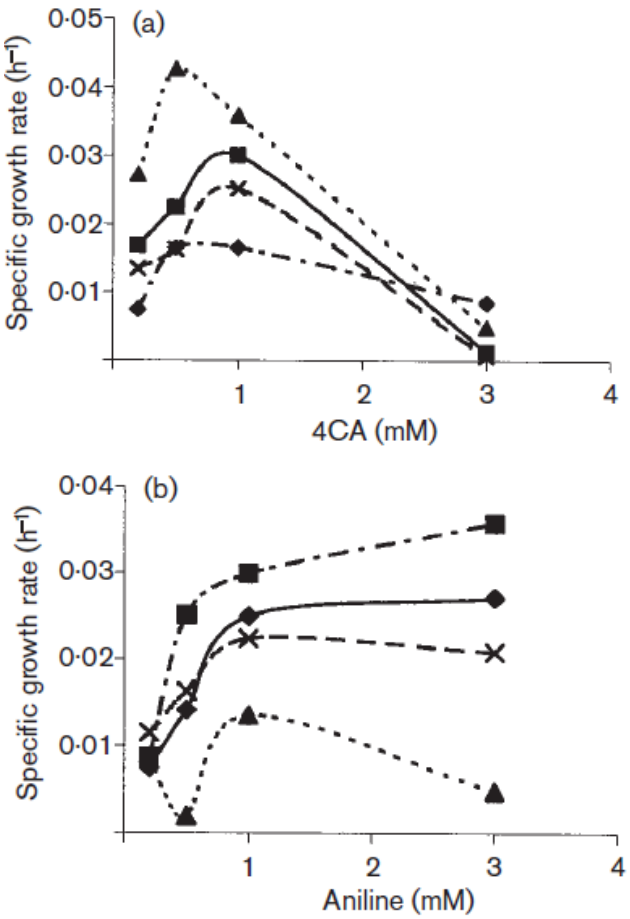


**Figure 3.** (a) Effect of 4CA concentration on the specific growth rate of the bacterial consortium on aniline. (b) Effect of aniline concentration on the specific growth rate of the bacterial consortium on 4CA. ◊, 0.2 mM; x, 0.5; mM; □, 1 mM; Δ, 3 mM. Data represent means of experimental values in duplicate.

**Table 2.** Catechol 1,2-dioxygenase activity of cell-free extract from consortium SB in comparison with other reports.

| Strain | Pregrown on | Catechol 1,2-dioxygenase activity [U (g protein)$^{-1}$]* | | Source |
|---|---|---|---|---|
| | | Catechol | 4-Chlorocatechol | |
| Consortium | Aniline + 4CA | 156 | 17.2 | This study |
| *P. acidovorans* CA28 | Aniline | 64 | 13 | Loidl et al. (1990) |
| | 3-Chloroaniline | 46 | 62 | |
| *Pseudomonas sp.* JL1 | Aniline | 22 | ND | Latorre et al. (1984) |
| | 2-Methylaniline | 1 | ND | |
| *Pseudomonas sp.* B13 | Benzoate | 177 | 20 | Dorn and Knackmuss (1978) |
| | 3-Chlorobenzoate | 640 | 172 | |

on aniline, only *P. putida* C could utilize 4CA as a growth substrate and release Cl2 ions (data not shown). On agar plates, all strains showed good growth on aniline, catechol and cis, cismuconic acid, but only *S. maltophilia* D grew on 4-chlorocatechol. An attempt to investigate the growth of *S. maltophilia* D in liquid basal medium containing 1 mM 4-chlorocatechol was not successful because dark brown polymeric material was produced during incubation,

making HPLC analysis difficult. The dark brown colour also prevented the measurement of optical density and chloride concentration. When *S. maltophilia* D cultures incubated in 4-chlorocatechol liquid medium for 21 days were plated on tryptic soy agar, growth was observed within 72 h, indicating that this organism tolerated or utilized 4-chlorocatechol.

## DISCUSSION

It has been reported that many micro-organisms are capable of degrading chloroanilines (Bollag and Russel, 1976; Helm and Reber, 1979; Reber et al., 1979; Surovtseva et al., 1980a, b; Zeyer et al., 1985). Swenson et al. (2000) showed that the artificial selection of microbial ecosystems for 3-chloroaniline degradation resulted in an increasingly well-adapted ecosystem for biodegradation. However, the authors did not report whether degradation was concomitant with dechlorination. In the present study, the defined bacterial consortium was able to degrade 4CA in the presence of aniline, which was essential for bacterial growth. It has been reported previously that many aerobic microbial cultures were unable to grow on chloroanilines as sole carbon and nitrogen (Helm and Reber, 1979; Reber et al., 1979; Surovtseva et al., 1980a, b). In this study aniline supported the growth of the bacterial consortium more effectively than 4CA, which was evident by higher mmax for aniline than for 4CA. Accumulated evidence supports the contention that if a micro-organism utilizes a choice of substrates sequentially, then the so-called 'richer' substrate (the one that supports the highest mmax) would be utilized first (Bull, 1985). Thus, in the present work, it was considered that aniline was the more readily available substrate, leading to the conclusion that a mixture of 1 mM aniline and 1 mM 4CA was used sequentially by the bacterial consortium. A longer lag phase was observed when 1 mM aniline and 1 mM 4CA were supplied as a mixture than when 1 mM aniline or 1 mM 4CA was supplied individually as growth substrates. The long lag phase, however, was followed by higher specific growth rate, indicating that an acclimation process such as induction or derepression of enzymes, mutation or genetic exchange, multiplication of the initially small populations of degrading organisms, preferential utilization of other organic compounds before the chemical of interest, or adaptation to the toxins or inhibitors present (Aelion et al., 1987; Ascon-Cabrera and Lebeault, 1993; Lewis et al., 1986; Wiggins et al., 1987) was happening during the lag phase, allowing the bacteria to survive on the mixture. Despite 100% utilization of the non-chlorinated parent compound, the degradation of 4CA was much slower than that of aniline, due to the presence of the Cl– substituent (Paris and Wolfe, 1987; Reber et al., 1979). There are two possible reasons for this incomplete dechlorination. First, it could be due to the formation of minor amounts of 5-chloro-2-hydroxymuconic semialdehyde (Loidl et al., 1990; Zeyer and Kearney, 1982), a meta-cleavage product of 4-chlorocatechol by the action of a catechol 2,3-dioxygenase (Hartmann et al., 1979), which gives a distinctive greenish yellow colour to the bacterial cultures growing on 4CA (Hartmann et al., 1979; Loidl et al., 1990; Sala-Trepat and Evans, 1971; Surovtseva et al., 1980a; Zeyer and Kearney, 1982). In the work reported here, the culture liquid became greenish-yellow during the first 7 days of incubation. However, assays for catechol 2, 3-dioxygenase activity were negative with either catechol or 4-chlorocatechol as substrates. Loidl et al. (1990) also reported that catechol 2,3-dioxygenase activity was not detectable when *Pseudomonas acidovorans* strain CA28 was grown on 4CA, despite the greenish-yellow colour of the culture filtrate. Zeyer and Kearney (1982) detected only micromolar concentrations of 2-hydroxy-5-chloromuconic semialdehyde in cultures of *Moraxella* sp. strain G growing on 2.5 mM 4CA concurrently with the appearance of the greenish-yellow colour. Therefore, it is tentatively concluded that in the present study, catechol 2, 3-dioxygenase was not the main enzyme involved in the degradation of 4CA, although putative 5-chloro-2-hydroxymuconic semialdehyde was detected in the filtrate. The second possibility is the accumulation of 4-chlorocatechol, which can inhibit further degradation of 4CA (Surovtseva et al., 1993). In the present study, the culture filtrate subsequently changed to dark brown in colour after 10 days incubation. This change reflects the accumulation of 4-chlorocatechol, as revealed by HPLC analysis, and suggests that further degradation of 4-chlorocatechol may be the rate-limiting step in the metabolism of 4CA by the bacterial consortium. In addition, it cannot be discounted that the dark brown colour was also the result of a non-enzymic conversion of aniline, 4-CA or 4-chlorocatechol to various oxidation products (Parris, 1980). For example, redox reactions can result in oxidative coupling of chloroanilines and chlorocatechols, whereas surface-catalysed reactions can result in aniline polymerization (Adriaens, 1997; Bachofer et al., 1975; Sjoblad and Bollag, 1981). In this study, unknown compounds were detected by HPLC-UV at retention times of 2.5 and 5.5 min. It was possible that these unidentified compounds were transformation products from 4CA, non-enzymatic oxidation products of 4-chlorocatechol (Parris, 1980), or modifications of 4CA to other chloroaromatic compounds. An example of the latter case was reported by Zeyer and Kearney (1982), who found 4, 49-dichloroazobenzene in the culture filtrate of *Moraxella* sp. strain G grown on 4CA. Enzyme assays of the bacterial consortium revealed the activities of catechol 1,2-dioxygenase on catechol and 4-chlorocatechol, which converted the compounds to cis, cis-muconic acid and 3-chloro-cis,cis-muconic acid, respectively. These results, supported by HPLC-UV detection of oxoadipic acid in the culture supernatant, suggested that the bacterial consortium degraded catechol and 4-chlorocatechol primarily by the activity of catechol 1, 2-dioxygenase following the ortho-cleavage pathway. The enzyme had a much slower

conversion rate of 4-chlorocatechol than of catechol, indicating lower preference for chlorinated substrates. Several workers (Dorn et al., 1974; Dorn and Knackmuss, 1978; Reber et al., 1979; Zeyer et al., 1985) have noted that the catechol 1, 2-dioxygenases that are found in benzoate-, phenol- or aniline-degrading micro-organisms are generally inefficient at processing the chlorinated analogue (Table 2). Therefore, it is probable that the catechol 1, 2-dioxygenase in the bacterial consortium crude extracts was induced mainly by aniline. We found that the addition of aniline enhanced 4CA utilization by the bacterial consortium. It was likely that aniline induced the enzymes involved in 4CA transformation, but did not support further metabolism of the chlorinated products. Reber et al. (1979) observed that in the presence of additional growth substrates, 4CA was rapidly converted to 4-chlorocatechol by *Pseudomonas multivorans* strain An1. Nevertheless, further metabolism of this intermediate was too slow to support growth. Inhibition by 4-chlorocatechol of the complete degradation of 4CA was also reported by Surovtseva et al. (1993). These authors concluded that the loss of enzymatic activity which catalyses isomerization of 3-chloro-cis, cis-muconic acid is an additional factor responsible for the lack of growth on 4CA. In an attempt to assess the role of each member of the bacterial consortium, their growth in monocultures was studied. All strains grew individually on aniline, but only *P. putida* C could grow on 4CA in liquid culture. Many cases have been reported for microbial com-munities capable of degrading environmental pollutants where none or only few of the community members could individually utilize the target compounds (Davison et al., 1994; Kardena, 1995; Lappin et al., 1985; Rozgaj and Glancer-Soljan, 1992; Wolfaardt et al., 1994). Senior et al. (1976) and Fauzi et al. (1996) suggested that the co-existence of secondary degraders in a consortium contri-buted to the stability of the system, making it more resistant to environmental stress. Ascon-Cabrera and Lebeault (1993) reiterated that a possible reason for the resistance of certain chemicals to degradation by single strains may be that no single organism has all the characteristics required for degradation of the chemical, whereas these characteristics might be found in microbial consortia. Among the members of the bacterial consortium, *P. putida* C exhibited the highest growth rate on aniline/4CA mixtures and the ability to grow on both aniline and 4CA.

Harder et al. (1977) demonstrated that the strain with the highest specific growth rate at the actual concentration of the growth-limiting nutrient out-competed all others, while Lendenmann and Egli (1998) proposed that under carbon limited conditions heterotrophic bacteria which are able to utilize a wide range of the carbon compounds can grow faster than those which exhibit a narrow substrate spectrum. Therefore, it can be expected that *P. putida* C would out-compete the other strains in the consortium. However, the specific growth rate of the consortium was lower than that of *P. putida* C, indicating that competition had occurred among the members of the consortium in utilizing an aniline/4CA mixture. All members of the consortium were able to grow on aniline, catechol and cis,cis-muconic acid as monocultures, but only *Stenotrophomonas maltophilia* D grew on 4-chlorocatechol. These results may suggest that the degradation of 4CA by the bacterial consortium in the presence of aniline was a result of a number of interactions. While all members were able to mineralize aniline, *P. putida* C, a member of Pseudomonads group I, which metabolize aromatic compounds through the ortho-cleavage pathways that converge to b-ketoadipate (Palleroni, 1984), is proposed to be the main degrader in the consortium which converts 4CA to 4-chlorocatechol. *S. maltophilia* D, the only strain capable of growing on 4-chlorocatechol, further utilized 4-chlorocatechol once it was produced from 4CA metabolism by *P. putida* C after aniline was completely utilized. *Comamonas testosteroni* has been noted by many workers for its capability to degrade aromatic compounds via the meta-cleavage pathway (Arai et al., 1999; Bae et al., 1997; Boon et al., 2000, 2001; Hein et al., 1998; Hollender et al., 1997). It was possible that the *C. testosteroni* B in the present study was able to degrade aniline and 4CA via the meta-cleavage pathway, but its activity was repressed by the metabolism via ortho-cleavage pathway of the other strains. The role of *Pseudomonas stutzeri* A in 4CA degradation was not clear, despite many reports on Flavobacterium strains as capable of degrading chloroaromatics (Alonso et al., 1997; Ederer et al., 1997; Lo et al., 1998; Ma¨nnisto¨et al., 1999; Obata et al., 1997). It was possible that this strain was maintained in the consortium simply by its ability to utilize aniline. Although 100% dechlorination was not achieved in suspended cultures, wider options to improve the process can be offered by a bacterial consortium. For example, the potential of *S. maltophilia* D to utilize 4-chlorocatechol may be enhanced by developing an immobilized culture system, which can be more supportive to slow-growing microorganisms (Bouwer, 1989; Shreve and Vogel, 1992) and less sensitive to the presence of toxic and inhibitory materials (Lee et al., 1994; Shi et al., 1995).

## Conclusion

*P. stutzeri* A, *C. testosterone* B, *P. putida* C and *S. maltophilia* D were enriched and individually isolated as 4-chloroaniline-utilizing bacteria effluent contaminated soil. This characteristic is intriguing, as it was necessary for other chloroaniline-degrading bacteria reported so far to have either aniline or other nutrient as an inducer or a cosubstrate to stimulate the degradation pathway. These bacterial isolates exhibited a good growth rate and high biodegradation efficiency towards 4-chloroaniline via a modified ortho-cleavage pathway as well as a comparatively good biodegradability towards other chloroanilines demonstrating their potential use in bioremediation. The

investigation of nutrient supplemen-tation provided prelim-nary information for each bacterial isolate, which may be useful for further bioremediation application of 4-chloro-aniline-contaminated sites.

## Conflict of Interest

The author(s) have not declared any conflict of interests.

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