Characterization of a soil-derived bacterial consortium degrading 4-chloroaniline

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A bacterial consortium comprising four different species was isolated from an Indonesian agricultural soil using a mixture of aniline and 4-chloroaniline (4CA) as principal carbon sources. The four species were identified as Chryseobacterium indologenes SB1, Comamonas testosteroni SB2, Pseudomonas corrugata SB4 and Stenotrophomonas maltophilia SB5. 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, although both compounds were eventually depleted from the culture supernatant. However, despite 100 % disappearance of the parent substrates, the degradation of 4CA was always characterized by incomplete dechlorination and 4-chlorocatechol accumulation. This result suggests that further degradation of 4-chlorocatechol may be the rate-limiting step in the metabolism of 4CA by the bacterial consortium. 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 [156 U (g protein)⁻¹] than for 4-chlorocatechol [17.2 U (g protein)⁻¹], indicating preference for non-chlorinated substrates. Members of the bacterial consortium were also characterized individually. All isolates were able to assimilate aniline. P. corrugata SB4 was able to grow on 4CA solely, while S. maltophilia SB5 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.

INTRODUCTION

One group of environmental pollutants which is considered to be particularly troublesome due to their toxic and recalcitrant properties is the chloroanilines (EEC, 1976; Federal Register, 1979). Chloroanilines are widely used in the production of dyes, drugs and herbicides (Kearney & Kaufmann, 1969, 1975; Latorre et al., 1984), and are also common metabolites of the microbial degradation of various phenylurea, acylanilide and phenylcarbamate herbicides (Bartha, 1968; Bartha & Pramer, 1970; Kaufman & Blake, 1973; Wallnofer, 1969). Environmental problems may arise from rather high concentrations of free chloroanilines as critical components of industrial effluents; these are generated by co-metabolic transformations, e.g. hydrolytic cleavage of acyl derivatives of chloroanilines or reduction of nitrochlorobenzenes (Latorre et al., 1984).

One way to remove chloroanilines from industrial effluents is through biodegradation, and many efforts have been made to isolate bacteria capable of degrading chlorinated anilines (Bollag & Russel, 1976; Loidl et al., 1990; Reber et al., 1979; Surovtseva et al., 1987, 1993, 1996; Zeyer & Kearney, 1982). However, most aerobic microbial cultures are unable to grow on chloroanilines as sole sources of carbon and nitrogen (Helm & Reber, 1979; Reber et al., 1979; Surovtseva et al., 1980a, b). Although some micro-organisms are capable of metabolizing chloroanilines (Brunbach & Reineke, 1993; Latorre et al., 1984; Loidl et al., 1990; Surovtseva et al., 1985, 1996; Zeyer & Kearney, 1982), further degradation of chlorocatechols, the most likely intermediate product of chloroaniline deamination (Helm & Reber, 1979; Latorre et al., 1984; Loidl et al., 1990; Paris & Wolfe, 1987; Reber et al., 1979; Surovtseva et al., 1980a, b; Zeyer et al., 1985), often becomes the rate-limiting step in their mineralization (Loidl et al., 1990; Reber et al., 1979; Surovtseva et al., 1980a, b).
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.

**METHODS**

**Enrichment and isolation.** Soil samples from an agricultural site in Lembang, West Java, Indonesia, were used to enrich a percolating soil column containing 200 ml basal medium: 0.45 g KH₂PO₄, 1.2 g Na₂HPO₄, 0.1 g MgSO₄·7H₂O, 0.05 g CaSO₄·2H₂O and 3.35 mg trace elements per litre (Zeyer & Kearney, 1982). The trace elements solution was composed of 1 mg each of FeSO₄·7H₂O and MnSO₄·H₂O; 0.25 mg each of (NH₄)₂MoO₄·4H₂O, Na₂B₄O₇·10H₂O, Co(NO₃)₂·6H₂O, CuCl₂·2H₂O and ZnCl₂; plus 0.1 mg NH₄VO₃. 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. Air was pumped continuously through the column at 100 l h⁻¹, which caused the medium to circulate through the soil. 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 subcultured onto solid basal medium containing the same substrates. The purity of the cultures was checked by plating them onto Nutrient Agar medium (Oxoid). The single isolates obtained were recombined into a bacterial consortium by making individual bacterial suspensions with equal OD₆₀₀, and 200 μl of each suspension was added to 50 ml fresh basal medium in a 250 ml flask for subsequent experiments.

**Bacterial identification.** The isolates chosen for further research were identified according to their phenotypic and genotypic characteristics. Preliminary characterization was based on colony morphologies on Tryptic Soy Agar (Oxoid) and Gram staining. Further identification was done using the API 20NE test (API, Basingstoke, UK), fatty acid methyl ester (FAME) analysis and 16S rRNA gene sequencing.

**API 20NE test.** Bacterial suspensions for the API test were prepared using isolates grown 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 a further 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).

**FAME analysis.** This was done in the NERC Centre for Environment and Hydrology (CEH), Oxford, UK. FAME extracts were prepared and analysed by gas chromatography as described by Thompson et al. (1993). Fatty acid peaks were named by the Microbial Identification System (MIS) software (Microbial ID, Newark, DE, USA) and isolates identified using the MIS ‘Aerobe Library’.

**16S rDNA 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 μl 10× reaction buffer (100 mM Tris/HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3 at 20 °C), 2.5 μl Taq DNA polymerase (Boehringer Mannheim), 1 μM upstream primer, 1 μM downstream primer, 200 μM of each dNTP (Promega) and PCR H₂O (Sigma-Aldrich) combined in a total volume of 100 μl. 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 Qiagen PCR Purification Kit (Qiagen), adjusted to 200 ng μl⁻¹, and sent to the Advanced Biotechnology Centre (Imperial College School of Medicine, London, UK) 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 to the most similar sequences in the GeneBank and EMBL nucleotide sequence databases based on percentage similarities. The 16S rDNA partial sequences obtained in this study were deposited in GenBank under accession numbers AY050493, AY050494, AY050495 and AY050496.

**Degradation assays.** All experiments were done aerobically in 250 ml flasks at 30 °C with constant shaking (120 r.p.m.). Growth was monitored spectrophotometrically by measuring culture turbidity at 600 nm. Depletion of target compounds (as measured by HPLC) and the liberation of Cl⁻ 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.

The effect of one substrate on the utilization of the other by the bacterial consortium was also analysed 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 (i.e. 0.2 mM aniline with 0.2, 0.5, 1 and 3 mM 4CA) were determined, and the same procedure was also applied to analyse the reciprocal effects of 4CA on aniline utilization.

**Determination of enzyme activities.** Cell-free extracts of the bacterial consortium grown on a mixture of 1 mM aniline and 1 mM 4CA were prepared according to Dorn & 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 twice through an Aminco French pressure cell (SLM Instrument) 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 (silica cuvettes, 10 mm path length), 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 μg ml⁻¹). The solution was mixed and the A₂₈₀ measured on a Lambda 15 UV Spectrophotometer (Perkin Elmer).

**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 μm) (Phenomenex). 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 NH₄H₂PO₄ (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 & Sanik (1957).

RESULTS

Enrichment and isolation

Bacterial growth in the percolating soil column was observed after 5 days, during which time the colour of the liquid percolating through the column changed from colourless 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 subculturing, four distinct isolates, designated as SB1, SB2, SB4 and SB5, were obtained and recombined into a bacterial consortium.

Identification

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 characteristics: those of isolate SB1 were orange and circular with diameter 2–6 mm; those of isolate SB2 were off-white and circular with diameter 1–3 mm; those of isolate SB4 were white and circular with diameter 3–7 mm; and those of isolate SB5 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) indologenes SB1, Comamonas testosteroni SB2, Pseudomonas corrugata SB4 and Stenotrophomonas maltophilia SB5.

Metabolic characterization of the bacterial consortium

In batch cultures the bacterial consortium was able to degrade 4CA in the presence of aniline (Fig. 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>API 20 NE</th>
<th>FAME</th>
<th>Partial 16S rDNA</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Most similar species*</td>
<td>% id†</td>
<td>Most similar species</td>
</tr>
<tr>
<td>SB1</td>
<td>Agrobacterium radiobacter 99-9</td>
<td>Flavobacterium balustinum 0-028</td>
<td>Chryseobacterium indologenes (M58773) 95-5 (1309)</td>
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<tr>
<td>SB2</td>
<td>Unidentified –</td>
<td>Comamonas testosteroni 0-546</td>
<td>Chryseobacterium gleum (M58772) 95-3 (1306)</td>
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<tr>
<td>SB4</td>
<td>Unidentified –</td>
<td>Comamonas testosteroni 0-81</td>
<td>Comamonas testosteroni (AB00796) 99-7 (1096)</td>
</tr>
<tr>
<td>SB5</td>
<td>Xanthomonas maltophilia 99-2</td>
<td>Not determined –</td>
<td>Pseudomonas agarici (D84005) 98-2 (1405)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pseudomonas corrugata (D84012) 98-0 (1400)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pseudomonas hibisciola (AB021405) 99-6 (1069)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stenotrophomonas maltophilia (X95923) 99-5 (1068)</td>
</tr>
</tbody>
</table>

*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 are obtained 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.
period (i.e. 1152 h), dechlorination stopped at a chloride concentration of 0·25 mM (see Fig. 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 compared to 18 h) and a higher specific growth rate (0·034 ± 0·004 h⁻¹) than when each substrate was supplied alone (0·027 ± 0·003 h⁻¹ for aniline and 0·014 ± 0·006 h⁻¹ for 4CA) (Fig. 2). When aniline and 4CA were supplied individually, the bacterial consortium had higher $\mu_{\text{max}}$ and $K_s$ for aniline (0·037 ± 0·006 h⁻¹ and 0·074 ± 0·006 g l⁻¹ respectively) than for 4CA (0·020 ± 0·006 h⁻¹ and 0·022 ± 0·005 g l⁻¹ respectively), i.e. 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 Fig. 3(a). 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 (Fig. 3b).

**Putative degradation pathway analysis**

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 min and 5·52 min.

Further investigation of the 4CA degradation pathway was made by determining catechol 1,2-dioxygenase 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 members of the bacterial consortium as monospecies cultures**

To assess the possible role of each member of the bacterial consortium in 4CA degradation, their ability to grow as monospecies cultures on 4CA in the presence of aniline was investigated. All of the strains grew on mixed 1 mM aniline

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**Fig. 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 (▲). Symbols represent means of experimental values in duplicate and lines were fitted to polynomial curves of the best order (3rd–5th) using Microsoft Excel 2002.

**Fig. 3.** (a) Effect of 4CA concentration on the specific growth rate of the bacterial consortium on aniline. (b) Effect of aniline concentrations on the specific growth rate of the bacterial consortium on 4CA. ●, 0·2 mM; ×, 0·5 mM; ■, 1 mM; ▲, 3 mM. Data represent means of experimental values in duplicate.
and 1 mM 4CA with specific growth rates ranging from 0.023 to 0.055 h⁻¹; Pseudomonas corrugata SB4 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 on aniline, only P. corrugata SB4 could utilize 4CA as a growth substrate and release Cl⁻ ions (data not shown). On agar plates all strains showed good growth on aniline, catechol and cis,cis-muconic acid, but only Stenotrophomonas maltophilia SB5 grew on 4-chlorocatechol. An attempt to investigate the growth of S. maltophilia SB5 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 SB5 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**

Many micro-organisms have been reported as capable of degrading chloroanilines (Bollag & Russel, 1976; Helm & Reber, 1979; Reber et al., 1979; Surovtseva et al., 1980a, b; Zeyer et al., 1985) but to our knowledge this study is the first report of 4CA degradation using a defined bacterial consortium. Swenson et al. (2000) showed that the artificial selection of microbial ecosystems for 3-chloroanilime 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 & 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 \( h_{max} \) 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 (i.e. the one that supports the highest \( h_{max} \)) 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 & 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 & 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 & 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 & Evans, 1971; Surovtseva et al., 1980a; Zeyer & 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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pregrown on</th>
<th>Catechol 1,2-dioxygenase activity [U (g protein)⁻¹]</th>
<th>Source</th>
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<tr>
<td></td>
<td></td>
<td><strong>Catechol</strong></td>
<td><strong>4-Chlorocatechol</strong></td>
</tr>
<tr>
<td>Consortium SB</td>
<td>Aniline + 4CA</td>
<td>156</td>
<td>17-2</td>
</tr>
<tr>
<td>P. acidovorans CA28</td>
<td>Aniline</td>
<td>64</td>
<td>13</td>
</tr>
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<td></td>
<td>3-Chloroaniline</td>
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<td>62</td>
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<tr>
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<td>22</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2-Methylaniline</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Pseudomonas sp. B13</td>
<td>Benzoate</td>
<td>177</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3-Chlorobenzoate</td>
<td>640</td>
<td>172</td>
</tr>
</tbody>
</table>

*1 U represents the conversion of 1 mmol substrate min⁻¹ at 30 °C. ND, Not determined.*
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 & Kearney (1982) detected only micromolar concentrations of 2-hydroxy-5-chloro-2-hydroxymuconic 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; Bachoer et al., 1975; Sjoblad & Bollag, 1981). In this study unknown compounds were detected by HPLC-UV at retention times of 2-5 min and 5-5 min. It was possible that these unidentified compounds were transformation products from 4CA, non-enzymic 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 & Kearney (1982), who found 4,4’-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 oxoacidic 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 & 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 maltovorans 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 enzymic 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. corrugata SB4 could grow on 4CA in liquid culture. Many cases have been reported of microbial communities 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 & Glancer-Soljan, 1992; Wolfardt et al., 1994). Senior et al. (1976) and Fauzi et al. (1996) suggested that the co-existence of secondary degraders in a consortium contributed to the stability of the system, making it more resistant to environmental stress. Ascon-Cabreria & 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. corrugata SB4 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 & 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. corrugata SB4 would out-compete the other strains in the consortium. However, the specific growth rate of the consortium was lower than that of P. corrugata SB4, 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 SB5 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. corrugata* SB4, a member of Pseudomonads group I, which metabolize aromatic compounds through the *ortho*-cleavage pathways that converge to β-ketoadipate (Palleroni, 1984), is proposed to be the main degrader in the consortium which converts 4CA to 4-chlorocatechol. *S. maltophilia* SB5, the only strain capable of growing on 4-chlorocatechol, further utilized 4-chlorocatechol once it was produced from 4CA metabolism by *P. corrugata* SB4 after aniline was completely utilized. *Comamonas testosterone* 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. testosterone* SB2 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 *C. indolgenes* SB1 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; Männistö 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* SB5 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 & Vogel, 1992) and less sensitive to the presence of toxic and inhibitory materials (Lee et al., 1994; Shi et al., 1995).

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Degradation and Mode of Action


