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ORIGINAL PAPER

Degradation of mixtures of phenolic compounds by *Arthrobacter chlorophenolicus* A6

Maria Unell · Karolina Nordin · Cecilia Jernberg · John Stenström · Janet K. Jansson

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Abstract In this study the chlorophenol-degrading actinobacterium, *Arthrobacter chlorophenolicus* A6, was tested for its ability to grow on mixtures of phenolic compounds. During the experiments depletion of the compounds was monitored, as were cell growth and activity. Activity assays were based on bioluminescence output from a luciferase-tagged strain. When the cells were grown on a mixture of 4-chlorophenol, 4-nitrophenol and phenol, 4-chlorophenol degradation apparently was delayed until 4-nitrophenol was almost completely depleted. Phenol was degraded more slowly than the other compounds and not until 4-nitrophenol and 4-chlorophenol were depleted, despite this being the least toxic compound of the three. A similar order of degradation was observed in non-sterile soil slurries inoculated with *A. chlorophenolicus*. The kinetics of degradation of the substituted phenols suggest that the preferential order of their depletion could be due to their respective pKa values and that the dissociated phenolate ions are the substrates. A mutant strain (T99), with a disrupted hydroxyquinol dioxygenase gene in the previously described 4-chlorophenol degradation gene cluster, was also studied for its ability to grow on the different phenols. The mutant strain was able to grow on phenol, but not on either of the substituted phenols, suggesting a different catabolic pathway for the degradation of phenol by this microorganism.

Keywords *Arthrobacter chlorophenolicus* · Bioremediation · 4-Bromophenol · 4-Chlorophenol · Mixed substrates · 4-Nitrophenol

Abbreviations:
4-BP 4-bromophenol
4-CP 4-chlorophenol
4-NP 4-nitrophenol

M. Unell · J. Stenström · J. K. Jansson (✉)
Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, 750 07 Uppsala, Sweden
e-mail: Janet.Jansson@mikrob.slu.se

K. Nordin
Department of Biochemistry and Biophysics, Stockholm University, 106 91 Stockholm, Sweden

K. Nordin · C. Jernberg
School of Life Sciences, Södertörn University College, 141 89 Huddinge, Sweden

C. Jernberg
Department of Laboratory Medicine, Karolinska Institute, Huddinge University Hospital, 171 76 Stockholm, Sweden

Introduction

Microbial biodegradation of pollutants is usually studied in the laboratory using single target compounds. This is an over-simplification since pollutants in nature most commonly occur as

mixtures, which can affect the degradation rates of the compounds (Kovárová-Kovar and Egli 1998). For example, the rate of degradation of some or all of the compounds in a mixture may be stimulated (Kim et al. 2002; Abuhamed et al. 2004; Baggi et al. 2004; Cejkova et al. 2005), or repressed (Abuhamed et al. 2004; Lovanh and Alvarez 2004; Cejkova et al. 2005) by the presence of other compounds. Degradation of several compounds may occur simultaneously (Heinaru et al. 2001; Kim et al. 2002), but in some cases one compound inhibits utilisation of a second compound until the first, preferred, substrate is metabolized. This is frequently the result of diauxie, where the enzymes catalysing degradation of the second compound are under transcriptional control and their expression is delayed until the preferred compound has been exhausted (Alexander 1999). This typically results in a slower growth of the culture during the induction period to the new substrate, which is seen as a transient plateau in the growth curve. Diauxic growth has been seen, for example, during sequential degradation of 4-methylphenol and 4-chlorophenol by *Comamonas testosteroni* JH5 (Hollender et al. 1994) and during growth of *Pseudomonas fluorescens* PC18 on a mixture of phenol and 4-methylphenol (Heinaru et al. 2001). The nature of pollutant interactions is thus variable, depending on the characteristics of the compounds and the degradation capacity of the involved microorganism(s).

Pollutants in mixtures may also impact the physiology and survival of the biodegrading organisms differently than single compounds. Phenols for example exert their toxicity by uncoupling oxidative phosphorylation (McLaughlin and Dilger 1980; Terada 1990; Escher et al. 1996), and formation of dimers between two differently substituted phenols can result in an uncoupling activity that is higher than the sum of the single compounds (Escher et al. 2001).

Our aim in the present study was to investigate the ability of a specific bacterium, *Arthrobacter chlorophenolicus*, to grow on mixtures of phenolic compounds. This bacterium was previously isolated from soil enriched with high concentrations of 4-chlorophenol (4-CP), and is also capable of growth on 4-nitrophenol (4-NP), 4-bromophenol (4-BP) and other *para*-substituted phenols as well as phenol, but not on compounds substituted in the *ortho* or *meta* position (Westerberg et al. 2000). *A. chlorophenolicus* has an unusually high substrate tolerance for degradation of 4-CP, 4-NP and phenol and the highest levels tolerated as sole carbon sources for growth are 2.7, 1.4 and 13 mM, respectively (Westerberg et al. 2000; Unell et al. 2007). The pathway for 4-CP degradation is unusual, via hydroxyquinol as a ring cleavage intermediate (Nordin et al. 2005), and this might be an explanation for the ability of *A. chlorophenolicus* to grow on high concentrations of this compound. The routes for degradation of 4-NP and phenol have not yet been elucidated. Therefore, one of the aims of this study was to use a mutant harbouring a non-functional 1,2 hydroxyquinol dioxygenase, disabling it to grow on 4-CP (Nordin et al. 2005), to study the role of this enzyme in the degradation of the other phenols.

Recently it was shown that 4-CP, 4-NP and phenol cause adaptive effects in the membrane of *A. chlorophenolicus*, the extent depending on the presence and nature of the substituent (Unell et al. 2007). The magnitude of this effect was correlated with the tolerance to the compound; the more toxic the phenolic compound, the stronger its effect on the cell membrane and the lower concentration tolerated before cell growth ceased. 4-NP was the most toxic to *A. chlorophenolicus*, followed by 4-CP and phenol. In this study we investigated whether these negative effects are also reflected in the order in which the different phenols are degraded when presented to *A. chlorophenolicus* as a mixture. We also studied the effect of the different phenols on each other's degradation and on the growth and activity of *A. chlorophenolicus*.

Materials and methods

Bacterial strains and culture conditions

The strains used were *A. chlorophenolicus* A6 wild type (A6 wt) (DSM12829^T) (Westerberg et al. 2000), *A. chlorophenolicus* A6L (chromosomally tagged with the firefly luciferase gene, *luc*) (A6L) (Elväng et al. 2001) and *A. chlorophenolicus* T99, a mutant with a transposon insertion in a gene encoding a hydroxyquinol dioxygenase, resulting in an inability to grow on or degrade 4-CP (Nordin et al. 2005). The strains were grown in GM minimal medium (Alexander and Lustigman 1966) as described previously

(Westerberg et al. 2000) with shaking (150 rpm) at 28°C. Yeast extract (0.1%, w/v) was added when higher cell densities were desired. Growth in pure cultures was monitored by optical density measurements (OD) at 600 nm or by direct microscopic cell counts. For conversion of OD-values to cell numbers, the factor 1.2×10^9 cells ml^{-1} per OD-unit of actively growing cells was used as determined by microscopic cell counts.

To study growth and degradation of phenols in pure cultures, A6 wt, A6L or T99 cultures were first grown to late log-phase (OD of 0.09–0.13) in GM medium with 150 μg/ml 4-CP as the sole carbon source, or 0.1% yeast extract as a carbon source for the T99 strain (late log phase OD of 0.3). The cells were washed, harvested by centrifugation (5000 × *g*, 22°C, 20 min) and resuspended in fresh GM medium to a suitable OD, whereupon the relevant phenols were added from stock solutions to triplicate cultures. The A6 wt and A6L cultures were incubated with the different phenols, with the total concentration of 4-CP + 4-NP varying between 0.7 and 1.5 mM in different relative proportions, and the concentration of phenol in the different experiments ranging from 0 to 1.3 mM.

Soil slurry set-up

Thirty grams of non-sterile soil (sandy loam, Ulleråker, Uppsala; characteristics of which were previously described (Stenberg et al. 1998)) were suspended in 300 ml 0.1 M phosphate buffer (pH 7.4) containing 4-CP, 4-NP and phenol to desired concentrations. A6 wt cultures were grown to stationary phase in GM medium with 1.95 mM 4-CP as the sole carbon source, harvested by centrifugation as described above, resuspended in PBS to 1/100 of the original volume and added to the soil slurries. Three replicate slurries were inoculated with *A. chlorophenolicus* A6 wt cells and three replicate slurries were uninoculated to serve as controls. The slurries were incubated at 28°C with shaking at 150 rpm.

Analysis of phenols

For quantitation of phenols in pure cultures, 2 ml samples were withdrawn and immediately chilled by submersion in –20°C ethanol. The frozen samples were stored at –20°C. Before extraction of phenolic compounds the samples were thawed and 100 μl 2-chlorophenol (0.97 mM) were added as an internal standard. The samples were then acidified by the addition of 10 μl concentrated sulphuric acid. Subsequently, 0.7 ml ethyl acetate was added and the samples were shaken. After separation of the phases, 0.4 ml of the organic phase was withdrawn for analysis by gas chromatography. Standard curves were routinely performed from known concentrations of the relevant compounds by the same procedure. Samples were analysed on a Varian 3800 gas chromatograph equipped with an 8200 autosampler and a flame ionization detector (Varian, Walnut creek, CA). The column was a 30 m db-1 ht (J&W scientific, Folsom, CA) with an inner diameter of 0.25 mm and a film thickness of 0.1 μm. The injection was splitless for 1 min and the column gas flow was kept constant at 0.8 ml/min. The temperature program was as follows: 1 min at 50°C, followed by an increase of 20°C/min to 70°C, then 3°C/min to 80°C, then again 20°C/min to 320°C which was held for 5.3 min. The detection limits were 0.01 mM for phenol and 4-CP and 0.02 mM for 4-NP.

Phenols were extracted from soil slurry samples (1.0 g) as previously described for extraction of 4-CP from soil (Elväng et al. 2001). The extraction efficiency of all phenols from spiked samples was greater than 90%. The compounds were quantified by gas chromatography, as described earlier (Elväng et al. 2001), except that a flame ionization detector was used instead of an electron capture detector, and the gas chromatograph was equipped with a Varian autosampler model 8200. The detection limits were 0.008 mM for 4-CP, 0.01 mM for phenol and 0.02 mM for 4-NP.

Luminometry

Bioluminescence emitted by the *luc*-tagged strain, A6L, was quantified by luminometry. A 10-μl aliquot of cells was removed from each culture, and mixed with 90 μl 0.11 mM citric acid buffer (pH 5.5) and incubated at room temperature for 5 min before addition of 10-μl luciferin (10 mM) (Promega, Madison, WI). The sample was mixed by brief vortexing and the light output as relative light units (RLU) was

immediately quantified for 30 s in a luminometer (MiniLumat LB 9506, Berthold, Bad Wilbad, Germany).

Kinetic modelling

The following equations were used to model the growth and degradation data:

Microbial exponential growth can be described as:

$$N = N_0 e^{\mu t} \quad (1)$$

where N is the concentration of active microorganisms at time t, N_0 the initial concentration of microorganisms and μ the specific growth rate. The rate of microbial substrate consumption then can be described as (Stenström 1989):

$$-\frac{dc}{dt} = qN_0 e^{\mu t} \quad (2)$$

where c is the substrate concentration at time t and q the specific microbial activity. The remaining concentration of substrate at a certain time is obtained by integrating Eq. 2:

$$c = c_0 - \frac{qN_0}{\mu}(e^{\mu t} - 1) \quad (3)$$

under the condition that the concentration of substrate equals c_0 when $t = 0$.

Results

Growth on and degradation of mixtures of phenols by *A. chlorophenolicus* A6

In three-component mixtures of 4-NP, 4-CP and phenol, 4-NP was almost completely depleted before the concentration of 4-CP started to decrease measurably, and 4-CP was degraded before phenol (Fig. 1a). On the other hand, when 4-CP and 4-bromophenol (4-BP) were added together, they were clearly degraded simultaneously (Fig. 1b). Similar results were obtained with different initial cell densities as well as with cells pre-grown on any of the phenolic compounds. The results of 13 different experiments with varying concentrations of 4-NP, 4-CP and phenol, all with an initial cell density set to an OD of 0.1, are summarized in Table 1.

The transition from growth on 4-CP to growth on phenol was sometimes accompanied by a transient levelling of the growth as measured by OD measurements (Fig. 1a). However, this plateau was not reflected in the rate of phenol removal, which was constant during this phase (Fig. 1a). Phenol was not fully removed even 24 h after the start of the experiments (data not shown). In order to investigate whether the slow growth on phenol was due to phenol being an inadequate inducer of the relevant catabolic enzymes, the cells were pre-grown on glucose and then transferred to phenol. Under these conditions, phenol still supported growth, similar to that obtained by cells pre-grown on 4-CP.

We also examined the pH of the medium during growth on the phenols to exclude any effects of acidification on degradation rates. Typically, the pH dropped marginally, from 7.25 initially to 7.0 after growth for 24 h on a mixture of 4-NP, 4-CP and phenol.

A. chlorophenolicus was also able to degrade mixtures of phenolic compounds in soil slurries. The order of degradation of 4-NP, 4-CP and phenol was the same in inoculated non-sterile soil slurries as in pure cultures (Fig. 2). There was no depletion of any of the phenolic compounds in the uninoculated control slurries (data not shown). However, in contrast to the pure culture results the onset of phenol degradation started before 4-NP and 4-CP were depleted.

Luciferase activity measurements

The luciferase-tagged strain, A6L, was used in some experiments to monitor the relative metabolic activity during degradation of the phenolic compounds. The OD and luciferase activity values were highly correlated during the entire course of 4-CP and 4-NP degradation when the concentration of total phenols was 2.30 mM or lower (Table 1). At higher concentrations of phenols no correlation was seen due to repression of luciferase activity. When 4-CP and 4-NP were present as sole carbon sources or in a binary mixture, the light output dropped immediately upon their depletion and stabilized at a lower level, suggesting that the cellular energy reserves necessary for bioluminescence production were also depleted at that point.

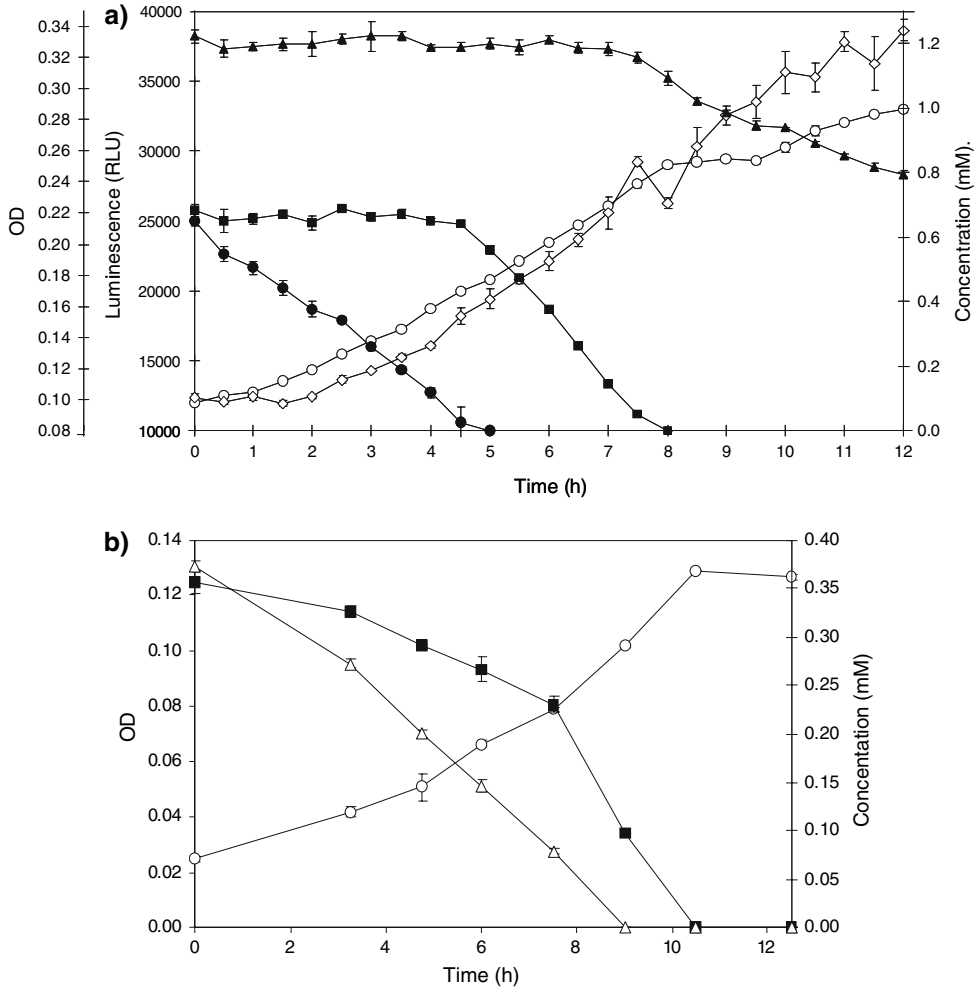

Fig. 1 Examples of depletion of mixtures of phenolic compounds by *Arthrobacter chlorophenolicus* A6L in pure culture. (**a**) Apparent sequential degradation of 4-NP and 4-CP, followed by degradation of phenol. (**b**) Simultaneous degradation of 4-CP and 4-BP. Open circles, OD; open diamonds, luminescence; filled circles, 4-NP; filled squares, 4-CP; filled triangles, phenol; open triangles, 4-BP. The error bars denote the standard deviation of the mean of three replicate samples ($n = 3$)

Growth on phenols by T99 mutant

With 4-NP or 4-BP as sole carbon source, the T99 mutant strain, with a disrupted hydroxyquinol dioxygenase gene, behaved the same way as when challenged with 4-CP (Nordin et al. 2005), i.e., no growth occurred and an orange pigment accumulated in the cultures, suggesting that the same catabolic route was used for degradation of these compounds. On the other hand, the T99 mutant grew at least as well as the wild type strain on phenol as the sole carbon source with a much shorter lag phase (Fig. 3).

Kinetics of degradation

Upon closer examination of the pure culture data (Table 1), we found that both 4-NP and 4-CP could individually and together support exponential growth of *A. chlorophenolicus* strains, as determined by plotting ln OD versus time, resulting in a straight line (a representative graph is shown in Fig. 4a).

To investigate if diauxie occurred in the transition between removal of 4-NP and 4-CP, the results were used from all 13 experiments, including both the wild type strain and the *luc*-tagged derivative to increase

Table 1 Mean values of the results of different degradation experiments (n = 3 with the coefficient of variation <3% in all cases)

A6 strain	c_0 4-NP (mM)[a]	c_0 4-CP (mM)[a]	c_0 phenol (mM)[a]	μ(h^{-1})	q (fmol $cell^{-1}$ h^{-1})	Y (=μ/q) (cell $fmol^{-1}$)	R^2 for OD versus luminescence
wt	0.773	0	0	0.148	1.127	0.132	N/A
wt	0	0.809	0	0.161	0.986	0.164	N/A
wt	0.256	0.374	1.150	0.165	0.871	0.190	N/A
wt	0.305	0.374	1.169	0.161	0.913	0.176	N/A
wt	0.557	0.618	0	0.133	0.992	0.134	N/A
wt	0.665	0.664	1.175	0.138	1.069	0.129	N/A
wt	0.660	0.773	1.150	0.125	0.856	0.146	N/A
L	0.739	0	0	0.141	1.029	0.137	0.887
L	0	0.806	0	0.144	0.882	0.163	0.971
L	0.381	0.427	1.302	0.148	0.880	0.168	0.985
L	0.453	0.619	0	0.134	0.830	0.162	0.947
L	0.649	0.681	1.221	0.124	0.892	0.139	0.983[b]
L	0.724	0.822	1.156	0.108	0.812	0.133	0.975[b]

[a] c_0 values as measured by gas chromatography

[b] Repression of luminescence seen initially (up to and including 1 h and 2.5 h, respectively), correlation calculated from points after repression ceased

N/A: Not Applicable

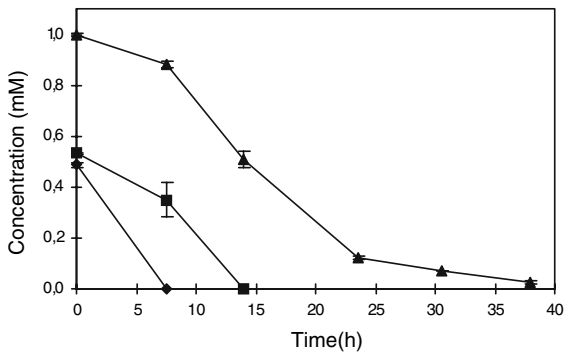

Fig. 2 Degradation of 4-NP, 4-CP and phenol in soil slurries by *A. chlorophenolicus* A6. Filled circles: 4-NP. Filled squares: 4-CP. Filled triangles: phenol. The error bars denote standard deviation of the mean of three replicate samples (n = 3)

the amount of data for analysis (Table 1). Data on the exponential microbial growth and on the associated degradation of 4-NP and 4-CP mixtures were fitted by non-linear regression to Eq. 1 and 3, respectively. The degradation data were fitted under the assumptions that 1) the 4-NP must be degraded completely before degradation of 4-CP starts (diauxie), or 2) 4-NP and 4-CP are degraded simultaneously, i.e., the sum of their molar concentrations were fitted to Eq. 3. The results obtained under the assumption of diauxic growth frequently either gave obviously erratic values of the parameters (e.g., negative values) or values of μ that significantly differed from those obtained by fitting growth data to Eq. 1. By contrast, results based on the assumption of simultaneous degradation gave reasonable parameter values and the values of μ obtained in most cases were similar to those obtained from the growth data. Furthermore, no effect on either OD or luciferase activity could be discerned at the time of an assumed diauxic substrate switch. Therefore, we conclude that diauxic growth did not occur and the data were analyzed as illustrated in Fig. 4a–c.

Different models were tested to describe the dependence of μ, q and Y on the initial concentrations of 4-NP, 4-CP and 4-NP + 4-CP. Significant regression coefficients ($P < 0.05$) were obtained according to the model: $\mu = 0.191 - 0.055[4\text{-NP}_0] - 0.035[4\text{-CP}_0]$ ($R^2 = 0.942$; $n = 7$) for the wild type and $\mu = 0.180 - 0.049[4\text{-NP}_0] - 0.040[4\text{-CP}_0]$ ($R^2 = 0.949$; $n = 6$) for A6L. The yield gave significant regression coefficients by the model: $Y = 0.178 - 0.098[4\text{-NP}_0]$ ($R^2 = 0.869$, $n = 7$) for the wild type and $Y = 0.170 - 0.080[4\text{-NP}_0]$ ($R^2 = 0.973$, $n = 6$) for A6L. The μ thus decreases with increasing initial concentrations of both 4-NP

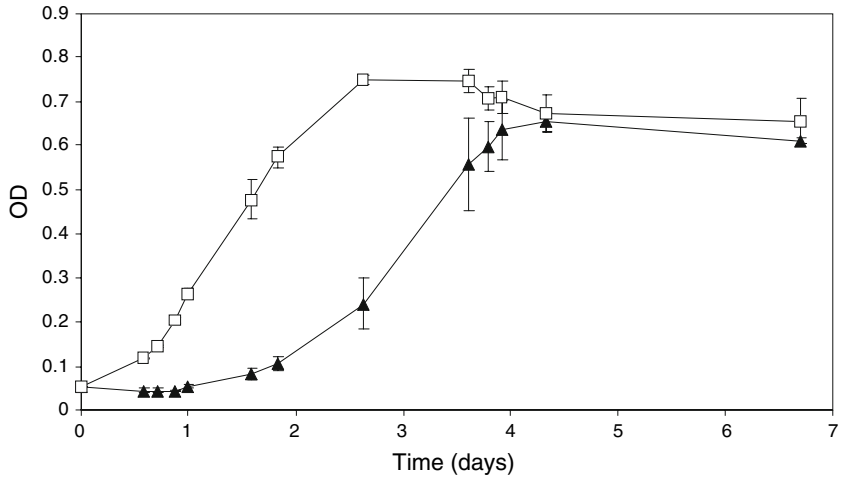

Fig. 3 Growth of *A. chlorophenolicus* A6 strains on 7 mM phenol. Open squares: mutant strain, T99, with a disrupted hydroxyquinol dioxygenase gene. Filled triangles: A6 wt. The error bars denote standard deviation of the mean of three replicate samples ($n = 3$)

and 4-CP, with sensitivity to this inhibiting effect by the two compounds not being significantly different.

When 4-CP or 4-NP were present as sole substrates, it was found that 4-CP gave a higher yield of biomass than 4-NP (Table 1). The stronger inhibiting effect of 4-NP on Y compared to 4-CP is also confirmed by the regression equation obtained, where 87% (for A6) and 97% (for A6L) of the variation in Y in the different experiments is explained by the initial concentration of 4-NP used.

When phenol, 4-CP and 4-NP were present together in a substrate mixture, phenol degradation was constant and progressed much more slowly than for the other two compounds (Fig. 1). Growth on phenol as the sole carbon source initially was exponential at high (>4 mM) initial concentrations (Fig. 3), with a μ of 0.036 ± 0.003 h^{-1} ($n = 3$), compared to that of cells growing on 4-CP and/or 4-NP ($\mu = 0.13$–0.17, see Table 1). *A. chlorophenolicus* often failed to degrade phenol at all at concentrations of approximately 1 mM and lower, a behaviour that was not seen for any of the other phenolic compounds tested. In mixtures, the presence of phenol did not affect the μ for 4-NP and 4-CP degradation (Table 1).

Discussion

Phenol is usually considered to be a less problematic pollutant than chlorophenol or nitrophenol, due to its lower toxicity. In fact, it is often used as a co-substrate with 4-CP to enhance degradation of the latter (Saez and Rittmann 1993; Bae et al. 1996; Hao et al. 2002; Kim et al. 2002). In a previous study, we determined that 4-NP was the most toxic to *A. chlorophenolicus* cells, followed by 4-CP and finally phenol (Unell et al. 2007). However, in this study, both 4-NP and 4-CP were degraded before phenol when the three compounds were added together in a mixture, reflecting a substrate preference uncorrelated to toxicity that, to our knowledge, has not been reported previously. This order of preference was observed regardless of which compound was used for pre-culturing the cells. In addition, the same substrate preference was observed when *A. chlorophenolicus* was inoculated into non-sterile soil slurries, suggesting that this substrate utilization pattern would also occur if the cells were inoculated into naturally contaminated sites containing an indigenous microflora.

Growth of the wild type strain on phenol was much slower than on the substituted phenols. When the cells were pre-grown on glucose they could still grow on phenol, suggesting that the slow growth on phenol was not due to lack of induction of the relevant catabolic enzymes. Furthermore, the slower growth on phenol was not due to exhaustion of growth factors in the medium or insufficient oxygen supply, as the pattern of phenol degradation was the same in all experiments even though phenol degradation started at different time points and at different cell densities. Also, the specific growth rate, μ, was unaffected by phenol in experiments where phenol was present together with 4-NP and 4-CP (Table 1), which likely is due to its low toxicity to the cells compared to 4-NP and 4-CP.

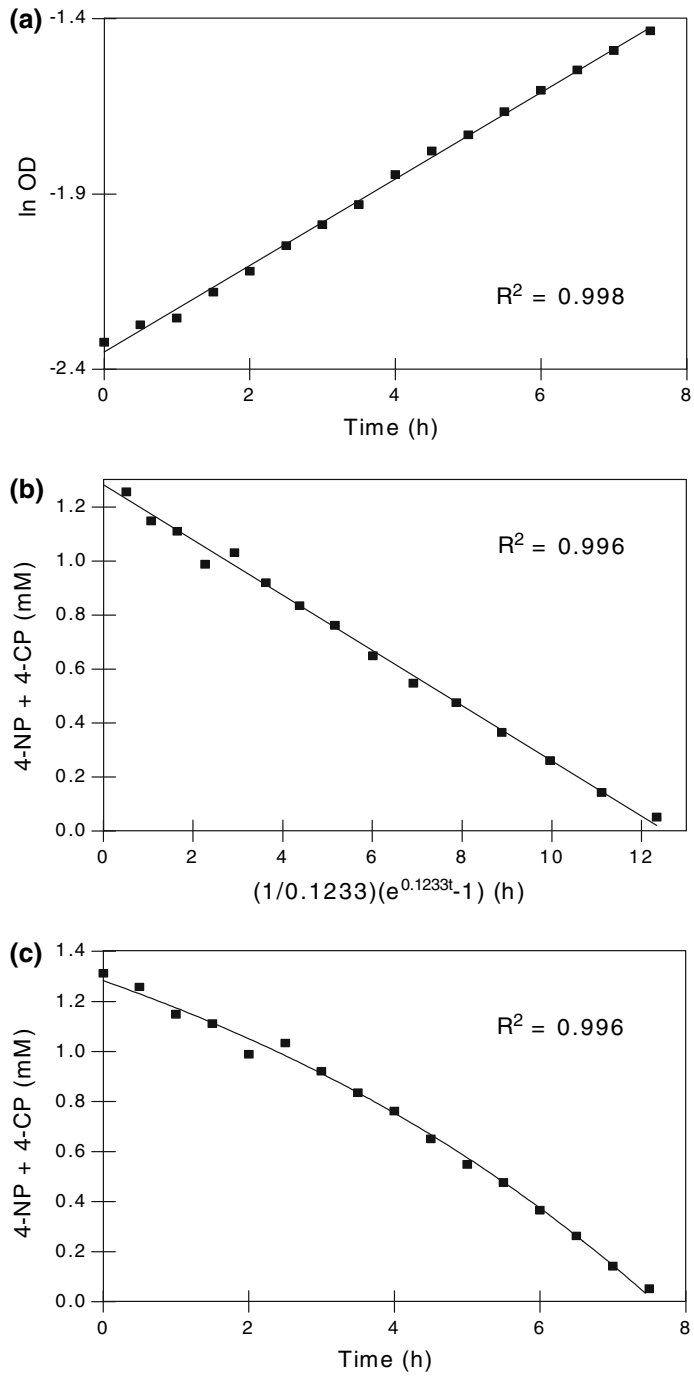

Fig. 4 (**a**–**c**) Example of the method used to fit growth and substrate consumption data. (**a**) Linear regression of ln OD versus time, the slope of which gives the value of μ. (**b**) Linearization of degradation data by plotting the remaining substrate concentration [4-NP + 4-CP] against $1/\mu$ (exp(μt)-1), with the value of μ obtained according to Fig. 4a inserted in Eq. 3. By this method the intercept of the linear regression line gives the value of [4-NP_0 + 4-CP_0] and the slope gives the value of qN_0 in Eq. 3. (**c**) A plot of the data in Fig. 4b plotted against t (squares) and the fit (line) to Eq. 3, with the value of μ obtained according to Fig. 4a and the values of c_0 and qN_0 obtained according to Fig. 4b

On the other hand, increasing initial concentrations of 4-CP and 4-NP decreased the specific growth rate (Table 1), reflecting the toxicity of 4-CP and 4-NP to *A. chlorophenolicus* A6. Although 4-NP was degraded prior to 4-CP, the kinetic data suggest that 4-NP had a stronger negative impact on the cells than did 4-CP. For example, the net energy gain decreased with increasing concentrations of 4-NP, as seen from the high negative correlation between Y and 4-NP. Furthermore, the substrate consumption per cell and hour (q) was higher for 4-NP as a sole substrate than for 4-CP as a sole substrate (Table 1, $P < 0.05$); i.e.

although the cells consumed 4-NP faster, the yield was lower. This indicates that the cells are more stressed when grown on 4-NP compared to 4-CP, consistent with previous reports that the cells grew on higher concentrations of 4-CP than 4-NP (Westerberg et al. 2000; Unell et al. 2007).

In a previous study (Unell et al. 2007) it was found that there was a greater change in the anteiso/iso ratio of the membrane fatty acids of *A. chlorophenolicus* cells when they were grown on 4-NP compared to 4-CP. Phenol caused the least effect on the anteiso/iso ratio, in accordance with the toxicity and lipophilic nature of the three compounds. 4-NP is probably a more potent uncoupler than 4-CP, and thus affects cells more negatively, in accordance with Escher et al. (1996) who found that both the type of substituent group on a phenol and the position of substitution had an impact on the uncoupling activity.

A possible explanation for the faster degradation of 4-NP in mixtures of 4-NP and 4-CP could be the different p*K*a values of the compounds, provided the substrate is their dissociated phenolate ions. At the pH of the experiments of 7.25, 55.7% of 4-NP is dissociated compared to only 0.7% of 4-CP. Also, according to this hypothesis, the obvious simultaneous degradation of mixtures of 4-CP and 4-BP could be caused by their similar availabilities due to their similar p*K*a values (9.41 and 9.37, respectively). The reason for the utilization of the phenolate ions as substrates is not known, but could be due to their preferential uptake across the cell membrane or specific requirements of the enzyme active site.

The results of this study indicate that 4-CP, 4-NP and 4-BP most probably are degraded by the same enzyme system, since diauxic growth was not seen for mixtures of these compounds, and the T99 mutant that is defective in degrading 4-CP also failed to degrade 4-NP and 4-BP. As the T99 mutant was rendered unable to grow on 4-CP by an insertion of a transposon into a hydroxyquinol 1,2-dioxygenase gene, the results suggest that cleavage of hydroxyquinol is a vital part of degradation of 4-NP and 4-BP as well as of 4-CP (Nordin et al. 2005). Hydroxyquinol is one of the central intermediates in the catabolic pathways of many different aromatic compounds (Travkin et al. 2006). The degradation pathways for 4-CP, 4-NP and 4-BP by *A. chlorophenolicus* A6 would thus converge, possibly explaining why their degradation kinetics are dependent on the sum of their molar amounts in mixtures. Other studies have also indicated possible convergence of degradation pathways for chlorophenols and nitrophenols (Xun et al. 1992; Leung et al. 1999). Furthermore, a preference for one aromatic compound over another even though the same degradation pathway is used has also been reported for other microorganisms. For example, *P. putida* F1 uses the same degradation pathway for toluene and phenol, but when challenged with a mixture of toluene and phenol, phenol degradation does not clearly start until almost all toluene has been removed from the medium (Reardon et al. 2000).

Interestingly, the mutant T99 strain could grow on phenol at least as well as the wild type. This suggests that another enzyme, or an additional pathway, is used by *A. chlorophenolicus* A6 to degrade phenol. Phenol is commonly degraded through catechol as the ring cleavage substrate, as reported for another *Arthrobacter* species (Karigar et al. 2006). However, crude cell extracts of phenol-induced *A. chlorophenolicus* A6 and T99 cells did not transform catechol (data not shown). Alternatively, the much shorter lag phase and more efficient growth displayed by T99 growing on phenol, compared to the wild type, might be due to downstream effects of the mutation or the mutation could affect the regulation of a phenol degradation enzyme resulting in its upregulation in the T99 mutant. It is outside the scope of this study to elucidate the phenol degradation pathway of *A. chlorophenolicus* A6, and we are conducting further research in this area.

Despite the indications of different catabolic pathways for substituted phenols versus phenol, degradation of phenol did not begin noticeably until 4-CP was almost depleted, indicating some sort of interaction between the compounds. This could be by a regulatory mechanism, or perhaps by the compounds competing for the same transport route into the cells. For example, transport level interactions between aromatic pollutants have been implicated in another study (Reardon et al. 2000). The fact that phenol degradation by *A. chlorophenolicus* A6 is non-exponential or does not always commence at all at low phenol concentrations, but is exponential at higher concentrations, could also indicate a concentration-dependent, passive transport of phenol into the cells.

In contrast to results in pure cultures, phenol degradation in soil slurries started before 4-NP and

4-CP were depleted. This difference could be due to participation of the indigenous soil microbiota in phenol degradation. Alternatively micro-scale niches in the soil environment may be present where the halogenated phenols were reduced sufficiently to enable localized onset of phenol degradation. The soil conditions could possibly also promote rapid adaptation of *A. chlorophenolicus* to the substrates present as reported previously for another phenol-degrading strain (Sarand et al. 2001).

The light output from the *luc*-tagged strain provided insight about the effect of phenolic substrates on the cells since light output from luciferase-tagged strains is correlated to their metabolic status and energy reserves (Jansson 2003). In previous studies, growth (OD) and luciferase activity from A6L were found to be highly correlated during exponential growth (Elväng et al. 2001; Backman and Jansson 2004). However, in the current study, luciferase activity initially was repressed at the higher concentrations of total phenols, emphasizing their toxic effect on suppressing cellular activity. The generation time was found to be slightly longer for the *luc*-tagged A6L strain than for the A6 wt (Table 1) and this could be due to the burden on the cells to express the luciferase enzyme or to positional effects of the *luc* gene insertion. It should be noted, however, that the ATP-consuming reaction by which *A. chlorophenolicus* A6L produces light is not initiated until the enzyme substrate, luciferin, is added exogenously (Elväng et al. 2001).

Conclusions

Arthrobacter chlorophenolicus displays an unusual substrate preference in degrading phenols in the order 4-NP > 4-CP > phenol, with the most toxic phenols first. The preferential depletion of one compound before another could be due to different proportions of dissociated ions in the case of the substituted phenols, when the dissociated ions are the substrate. The results of this study highlight the need for further research on substrate preferences and the impact of environmental factors, such as pH, on uptake of phenolic pollutants into degrading cells for bioremediation purposes. The study also sheds light on how chemically similar compounds may be metabolised by different degradation pathways in the same organism, as is most probably the case for the degradation of phenol vs. substituted phenols by *A. chlorophenolicus*. For the latter, hydroxyquinol is the common ring cleavage substrate (Nordin et al. 2005), whereas for phenol another pathway is employed.

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