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ORIGINAL PAPER

Induction of enzymes of 2,4-dichlorophenoxyacetate degradation in *Burkholderia cepacia* 2a and toxicity of metabolic intermediates

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Abstract *Burkholderia cepacia* 2a inducibly degraded 2,4-dichlorophenoxyacetate (2,4-D) sequentially *via* 2,4-dichlorophenol, 3,5-dichlorocatechol, 2,4-dichloromuconate, 2-chloromuconolactone and 2-chloromaleylacetate. Cells grown on nutrient agar or broth grew on 2,4-D-salts only if first passaged on 4-hydroxybenzoate- or succinate-salts agar. Buffered suspensions of 4-hydroxybenzoate-grown cells did not adapt to 2,4-D or 3,5-dichlorocatechol, but responded to 2,4-dichlorophenol at concentrations <0.4 mM. Uptake of 2,4-dichlorophenol by non-induced cells displayed a type S (cooperative uptake) uptake isotherm in which the accelerated uptake of the phenol began before the equivalent of a surface monolayer had been adsorbed, and growth inhibition corresponded with the acquisition of 2.2-fold excess of phenol required for the establishment of the monolayer. No evidence of saturation was seen even at 2 mM 2,4-dichlorophenol, possibly due to absorption by intracellular poly-β-hydroxybutyrate inclusions. With increasing concentration, 2,4-dichlorophenol caused progressive cell membrane damage and, sequentially, leakage of intracellular K^+, P_i, ribose and material absorbing light at 260 nm (presumed nucleotide cofactors), until at 0.4 mM, protein synthesis and enzyme induction were forestalled. Growth of non-adapted cells was inhibited by 0.35 mM 2,4-dichlorophenol and 0.25 mM 3,5-dichlorocatechol; the corresponding minimum bacteriocidal concentrations were 0.45 and 0.35 mM. Strain 2a grew in chemostat culture on carbon-limited media containing 2,4-D, with an apparent growth yield coefficient of 0.23, and on 2,4-dichlorophenol. Growth on 3,5-dichlorocatechol did not occur without a supplement of succinate, probably due to accumulation of toxic quantities of quinonoid and polymerisation products. Cells grown on these compounds were active towards all three, but not when grown on other substrates. The enzymes of the pathway therefore appeared to be induced by 3,5-dichlorocatechol or some later metabolite. A possible reason is offered for the environmental persistence of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T).

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Abbreviations

2-CL	2-Chlorodienelactone
2-CM	2-Chloromaleylacetate

3,5-DCC	3,5-Dichlorocatechol
2,4-DCM	2,4-Dichloromuconate
2,4-DCP	2,4-Dichlorophenol
2,4-D	2,4-Dichlorophenoxyacetate
2,4,5-T	2,4,5-Trichlorophenoxyacetate
DMG	3,3′-Dimethylglutarate
4-HB	4-Hydroxybenzoate
α-KG	α-Ketoglutarate
MBC	Minimum bacteriocidal concentration
MCPA	4-Chloro-2-methylphenoxyacetate
MIC	Minimum inhibitory concentration
P_i	Inorganic phosphate

Introduction

The degradation pathways for the herbicides 2,4-dichlorophenoxyacetate (2,4-D) and 4-chloro-2-methylphenoxyacetate (MCPA) were largely elucidated in *Pseudomonas* sp. (Evans et al. 1971a, b; Gaunt and Evans 1971a, b) and *Arthrobacter* (Bollag et al. 1968a, b; Tiedje et al. 1969; Tiedje and Alexander 1969; Sharpee et al. 1973). The 2,4-D pathway is inducible and proceeds *via* 2,4-dichlorophenol (2,4-DCP), 3,5-dichlorocatechol (3,5-DCC), *cis, cis*-2,4-dichloromuconate (*cis, cis*-2,4-DCM), 2-chlorodienelactone (2-CL) and 2-chloromaleylacetate (2-CM). MCPA follows an analogous route.

2,4-D/α-ketoglutarate (α-KG) dioxygenase, which cleaves 2,4-D to yield 2,4-DCP and glyoxylate, was first demonstrated in *Cupriavidus necator* (= *Ralstonia eutropha* = *Alcaligenes eutrophus*) JMP134 by Fukumori and Hausinger (1993a, b). 2,4-DCP hydroxylase (EC 1.14.13.20) was first purified from *Burkholderia cepacia* strain 2a (Beadle and Smith 1982; Beadle et al. 1984). 3,5-DCC 1,2-dioxygenase, 2,4-DCM cycloisomerase and 2-CL hydrolase were originally described by Evans and Alexander and their co-workers (see above); subsequently Pieper et al. (1988) characterised the ring-fission oxygenase and the cycloisomerase of *C. necator* JMP 134 as Type II enzymes, metabolizing chlorinated substrates more rapidly than their unchlorinated analogues.

The most notable bacterium amongst the many 2,4-D-degraders that have now been isolated is *C. necator* JMP134, which contains the degradative plasmid pJP4 (Fisher et al. 1978; Don and Pemberton 1981), which bears *tfdA* (Streber et al. 1987), *tfdB,C,D,E* and *F* (Don et al. 1985), respectively specifying the five enzymes described above, and a putative 2-CL isomerase. Control of gene expression in these bacteria is incompletely understood, partly because the antimicrobial properties (of 2,4-DCP and 3,5-DCC), or instability (of the post-ring-fission products) has hampered a systematic study of their role in induction. Two genes act to control the pathway: *tfdR* is a negative regulator for *tfdA* and *tfdCDEF* (Kaphammer et al. 1990), and *tfdS* is the repressor-activator gene of *tfdB* (Kaphammer and Olsen 1990). Filer and Harker (1997) produced genetic evidence to suggest that the inducer is *cis, cis*-2,4-DCM.

Burkholderia cepacia strain 2a has also received extensive study. Besides 2,4-DCP hydroxylase, 2,4-D/α-ketoglutarate (α-KG) dioxygenase was purified by Poh et al (2001), and a chloride channel protein (ClC) has recently been shown to be essential for 2,4-D dissimilation (Sebastianelli and Bruce 2007). The organism carries the plasmid pIJB1, within which the completely-characterised transposon *Tn5530* bears the 2,4-D pathway genes (Poh et al. 2002), including a gene corresponding with *tfdR*, but not *tfdS*, and a gene for a leucine-responsive regulatory factor (*lrp*). These differences could signal dissimilarities between the mechanisms of expression control between *C. necator* JMP 134 and *B. cepacia* 2a. The present paper describes the 3,5-DCC dioxygenase, 2,4-DCM cycloisomerase and 2-CL hydrolase of strain 2a (briefly outlined earlier by Poh et al. 2002), and the induction of the initial three enzymes by growth in the presence of 2,4-D, 2,4-DCP and 3,5-DCC. Evidence is given of the toxicity to the organism of 2,4-DCP, and the manner in which it prevents enzyme induction.

Materials and methods

Organisms

Strain 2a, originally identified as *Acinetobacter* at the National Collection of Industrial and Marine Bacteria, Aberdeen, UK and latterly re-identified as *B. cepacia* (Poh et al. 2001), was maintained as a lyophilisate and by routine serial subculture on 2,4-D-salts agar (Beadle and Smith 1982). *Pseudomonas* NCIMB 9340 was obtained from the source collection.

Batch culture

Batch cultures were grown in salts medium containing 2.26 mM Na-2,4-D (Beadle and Smith 1982), or 4-hydroxybenzoate (4-HB), glutamate or succinate (all 10 mM) as sole carbon sources. Growth from one 24–48 h-old slope was inoculated per 250 ml of the appropriate medium in 1 l conical flasks, and incubated with agitation at 24°C for 1–2 days.

Continuous culture

2,4-DCP and 3,5-DCC solutions were sterilised by Millipore filtration before adding to the autoclaved salts medium. A baffled 10 l vessel on a Model 1/1000 fermenter (LH Engineering Ltd., Stoke Poges, UK), fitted with an internal weir and containing 7.5 l of the relevant medium, each 18.1 mM in elemental carbon content, was inoculated with 500 ml of culture in late exponential phase, then operated batchwise at 24°C with agitation at 900 rev min^{-1} and aeration at 1 l min^{-1}, giving an absorption rate by sulphite oxidation (Cooper et al. 1944), of 300 mmol O_2 h^{-1}. Control of pH at 6.5 was by automatic addition of 0.5 M-HCl or $NaHCO_3$.

When the culture neared maximal turbidity, a continuous medium supply was begun at 440 ml h^{-1} ($D = 0.055$ h^{-1}), and antifoam (3 drops of polypropylene glycol 2000; Shell Chemical Co. Ltd., London) was added every 4.5 h to prevent cell flotation and washout. Under these conditions, the O_2 requirement with all media for complete substrate oxidation was approximately 7 mmol h^{-1}, and the oxygen tension was routinely >90% saturation. When carbon sources were changed, culture samples were collected at 4°C after four replacement times following the input of new medium, to allow removal of the original culture components (Pirt 1975). Cells were centrifuged at 10 000 g for 10 min, washed once in buffer and used directly or stored at −20°C.

Determinations

2,4-D ($E_{mM} = 2.21$) was estimated by measurement of $A_{283\ nm}$. 2,4-DCP was determined by the method of Lacoste et al. (1959), and 3,5-DCC by the procedure of Mitchell (1924). Protein was determined by biuret assay.

Total cell counts, turbidimetry and dry weight determinations

Total cell counts were performed in a Thoma chamber (Hawksley Ltd., London, UK). Cell dry weights were determined on samples washed in water and dried at 105°C to constant weight, and were correlated with $OD_{540\ nm}$ measurements of cell suspensions.

Buffered cell incubations and respirometry

Substrate consumption by shaken cell suspensions (28 ml; 0.23 mg dry weight ml^{-1}) in 50 mM Na-3,3′-dimethylglutarate (DMG) buffer, pH 6.5 (Dawson et al. 1969) was followed at 24°C after addition of 10 µmol of substrate to initiate reaction. Periodically, 5-ml portions were withdrawn, acidified (0.5 ml of 0.5 M H_2SO_4), and centrifuged. Supernatants were assayed for residual substrate.

In parallel experiments, oxygen uptakes were followed by conventional Warburg respirometry (Umbreit et al. 1964) at 24°C, in a Model V respirometer (B. Braun GmbH, Melsungen, Germany). The main compartment contained 2.7 ml of cell suspension and the centre well 0.2 ml of 20% (w/v) KOH. After tipping substrate (0.1 ml) from the sidearm, oxygen uptake was followed until substrate-dependent respiration ceased.

Enzyme assays

2,4-DCP hydroxylase was extracted from 0.5 g of packed cells washed in 67 mM phosphate buffer, pH 7.8, and assayed spectrophotometrically (Beadle and Smith 1982).

3,5-DCC dioxygenase was released from packed cells (0.5 g) washed in 50 mM Na-DMG or 67 mM phosphate buffer, pH 6.5, by disruption in 10 ml of the same buffer containing 1 mM-cysteine HCl and 1 mM-$FeSO_4$ ("stabilising buffer"). After centrifugation, the supernatant was assayed for at 30°C in a Clark electrode containing 2.6 ml of buffer and

0.4 ml of cell extract (2.6 mg of protein). Oxygen consumption was followed for 4 min before injecting 25 µl of 10 mM-3,5-DCC.

The formation of post-ring fission products was followed in 1-cm quartz cuvettes by repetitive scanning between 190 and 350 nm in a recording spectrophotometer (Model 402; Perkin Elmer, Beaconsfield, UK) at 30°C, against no-substrate controls. The assay system contained 0.04 ml of cell extract (0.67 mg of protein) made to 3.0 ml with stabilising buffer. Reactions were initiated by adding 25 µl of 10 mM-3,5-DCC.

Extracts of 2,4-D-grown *Pseudomonas* NCIMB 9340 cells were used to prepare authentic 2,4-DCM from 3,5-DCC (Gaunt and Evans 1971b). After ultrafiltration (cut-off mol. wt. >10,000) to remove protein, and ether-extraction to remove residual 3,5-DCC, the reaction yielded 0.16 mM 2,4-DCM.

Minimum inhibitory concentrations (MICs) and growth inhibition

MICs of 2,4-DCP and 3,5-DCC were determined in tubes containing 4-HB-salts medium, supplemented with 2,4-DCP or 3,5-DCC in graded concentrations from 0.05 to 1 mM and inoculated with 2×10^7 organisms ml^{-1} from overnight cultures in the same medium minus 2,4-DCP or 3,5-DCC. Tubes were inspected for growth after incubation for 2 days. Approximate minimum bacteriocidal concentrations (MBCs) were determined by streaking samples onto nutrient agar from tubes showing no growth, and examining after a further 2 days.

Inhibitory effects of 2,4-DCP and 3,5-DCC were investigated using cultures growing exponentially in 4-HB-salts medium. When cultures reached $OD_{540\ nm} = 0.3$, 2,4-DCP or 3,5-DCC solution was added, up to 0.5 mM. After following growth for 4 h, total cell counts were performed, and centrifuged culture supernatants were assayed for residual 2,4-DCP or 3,5-DCC.

2,4-DCP uptake and leakage of intracellular components

2,4-DCP (up to 0.5 mM) was added to shaken suspensions of 4-HB-grown cells (0.63 mg dry weight ml^{-1}) at 24°C in 25 ml of Na-DMG buffer, pH 6.5 (except for following K^+ release). 2,4-DCP uptake by cells was assessed after 5 min contact, and periodically thereafter for 6 h. Samples were centrifuged and the 2,4-DCP content of the supernatants was determined. Values from the initial samples were used to construct an absorption isotherm.

K^+ release was measured in Tris-DMG buffer, pH 7.0, using a valinomycin electrode (Phillips Type 1S 540-K; Lambert and Hammond 1973). After 30 min, suspensions were centrifuged and the cells were disrupted by ultrasonic irradiation to liberate remaining unbound K^+ for determination. Leakage of inorganic phosphate (P_i) and (bound) ribose was followed by periodic centrifugation of 3-ml samples at 10,000*g* for 10 min and assay of supernatants for P_i by semidine (Dryer et al. 1957), and for ribose equivalents by the orcinol method (Herbert et al. 1971). After 6 h, suspensions remaining were centrifuged; supernatants were extracted with 2×5 ml of ether to remove 2,4-DCP, and $A_{260\ nm}$ values of the aqueous phase were read against a buffer blank as a measure of the release of material absorbing light at 260 nm ("260 nm-absorbing material"; presumptive pool nucleotides).

Results

The lower pathway: 3,5-DCC 1,2-dioxygenase, 2,4-DCM cycloisomerase and 2-CL hydrolase

3,5-DCC 1,2-dioxygenase of strain 2a is composed of two subunits (Carrington 1994) of 25.7 kD (deduced value; Poh et al. 2002), and resembles the corresponding enzyme of *Pseudomonas* sp. NCIMB 9340 (Evans et al. 1971a; Gaunt and Evans 1971b) in requiring Fe^{2+} and cysteine (or less effectively, GSH). Activity was stable at −20°C in unsupplemented cell extract for >1 month; detectable over a broad pH range, with an optimum in phosphate buffer of 6.5, and maximal at 35°C and at 0.08 mM 3,5-DCC, progressively declining with higher substrate concentrations. Crude cell extracts oxidized 3,5-DCC (most rapidly); 3-methyl-5-chloro-, 3-chloro- and 4-chlorocatechol, catechol and protocatechuate. The reaction rate with 3,5-DCC was 2.8 times greater than with catechol, consistent with the identity of the ring-fission enzyme as of Type II, and the absence of Type

I activity (Pieper et al. 1988). Only the activity towards protocatechuate was unaffected by supplementation with Fe^{2+} and cysteine, indicating the presence of protocatechuate 3,4-dioxygenase, probably co-induced because the final two steps in chlorocatechol degradation are catalysed by enzymes of the β-ketoadipate pathway (Schlömann 1994).

Crude extract dialysed overnight against stabilising buffer oxidised 0.25 µmol of 3,5-DCC with consumption of 0.25 µmol O_2 to produce 0.23 µmol of 2,4-dichloromuconate in 92% yield, identified from its uv spectrum (Fig. 1: $\lambda_{max} = 268$ nm: $E_{mM} = 14.8$; Tiedje et al. 1969). Successive treatment with acid, alkali, and acid again yielded in turn 2-CL in 92% yield ($\lambda_{max} = 281$ nm: $E_{mM} = 17.1$; Evans et al. 1971a) and 2-CM in 76% yield ($\lambda_{max} = 253$ in alkali: $E_{mM} = 9.47$; Tiedje et al. 1969), the absorbance disappearing on reacidification. For comparison, extracts of 2,4-D-grown *Pseudomonas* NCIMB 9340

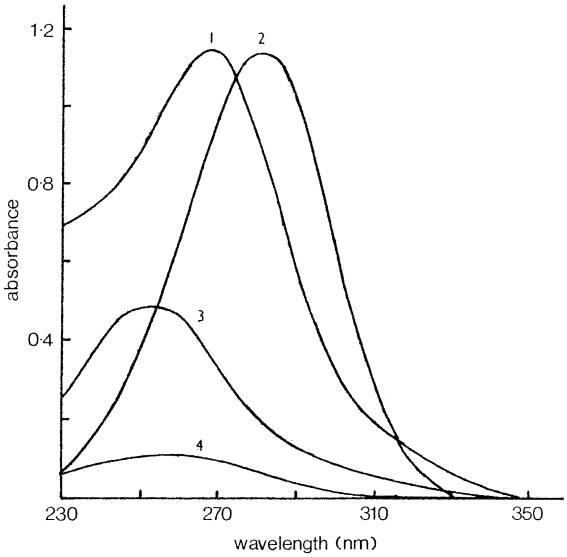

Fig. 1 UV spectra of ring-fission products derived from 3,5-DCC. 2,4-Dichloromuconate was produced by incubating cell extract of 2,4-D-grown 2a (40 µl; 0.67 mg of protein) made to 3.0 ml with DMG stabilising buffer in 1-cm quartz cuvettes. The reaction was initiated by adding 0.25 µmol of 3,5-DCC and monitored by repetitive scanning between 190 and 350 nm in a recording spectrophotometer (Model 402; Perkin Elmer) at 30°C, against a no-substrate control. Spectra: (1) Incubation mixture at the end of the reaction, which was treated sequentially as follows: (2) deproteinized with 10% (w/v) trichloracetic acid (0.3 ml); pH 1; (3) adjusted to pH 13 with 0.1 ml of 10 M NaOH; (4) readjusted to pH 1. In a separate experiment, addition of $MnSO_4$ (1 µmol) to the above enzyme incubation mixture resulted after 120 min in a spectrum similar to (3)

cells incubated under the conditions of Evans et al. (1971a) gave these products in 100, 100 and 81% yield respectively. In 67 mM phosphate buffer, pH 6.5, cell extracts of strain 2a also generated a minor, pink-brown product from 3,5-DCC, presumably the corresponding quinone and polymerisation products thereof.

Incubated with cell extract (0.67 mg of protein) supplemented with 0.33 mM Mn^{2+} for 2,4-DCM cycloisomerase (Evans et al. 1971a), 3,5-DCC yielded an absorbance spectrum of λ_{max} shifting through ~260 nm to 253 nm after 120 min, consistent with initial conversion to 2,4-DCM, then *via* 2-CL to 2-CM (yield, 84%; Fig. 1). *cis, cis*-2,4-DCM, prepared enzymically from 3,5-DCC using strain NCIMB 9340 and added to Mn^{2+}-supplemented cell extract of strain 2a, likewise yielded 2-CM, in 100% yield.

Preliminary induction studies: toxicity of intermediates to cells

Neither strain 2a nor *Pseudomonas* NCIMB 9340 cultured on nutrient agar grew on 2,4-D-salts unless first passaged on succinate-salts agar. Enzyme induction was therefore initially investigated by incubating 2,4-D, 2,4-DCP or 3,5-DCC with buffered suspensions of cells grown batchwise on succinate-, glutamate- or 4-HB-salts medium. Strain 2a failed to respond to either 2,4-D or 3,5-DCC even by 12 h, but began to dissimilate 2,4-DCP after 1.5–4 h at concentrations <0.4 mM (Fig. 2), though subsequently remaining inactive towards either 2,4-D or 3,5-DCC. The toxicity of the intermediates was investigated in greater detail.

2,4-D gave no clear MIC for *B. cepacia* 2a. Fairly rapid growth ($t_D = 7$ h) occurred at concentrations up to 2.26 mM: with further increases growth gradually became slower. MICs for 2,4-DCP and 3,5-DCC in 4-HB-salts media were lower, at 0.35 mM and 0.25 mM respectively; the corresponding MBCs were 0.45 and 0.35 mM. Added to organisms in exponential phase in 4-HB-salts ($t_D = 84$ min), 2,4-DCP and 3,5-DCC depressed the growth rate by 50% at 0.23 and 0.10 mM respectively (Fig. 3). Cells treated with 3,5-DCC acquired the pink-brown appearance mentioned above. Assay of culture supernatants revealed that cells had not begun to metabolise 2,4-DCP or 3,5-DCC after 3.5 h of cell contact.

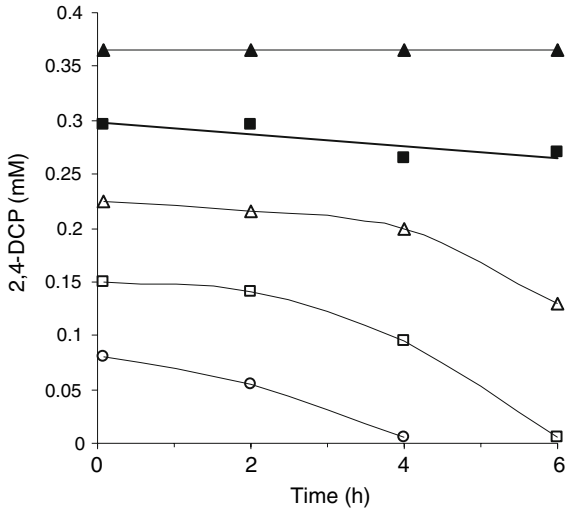

Fig. 2 Dissimilation of 2,4-DCP by suspensions of 4-HB-grown cells of *B. cepacia* 2a. Incubations were sampled at timed intervals and assayed for 2,4-DCP as described under Fig 4. 2,4-DCP concentrations (mM): ▲, 0.5; ■, 0.4; Δ, 0.3; □, 0.2; ◯, 0.1

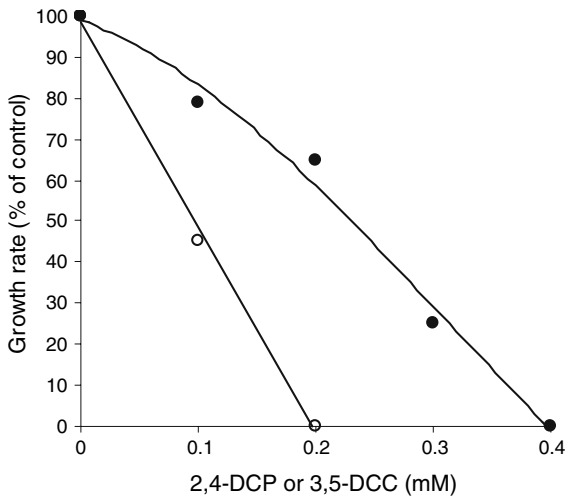

Fig. 3 Effects of 2,4-DCP and 3,5-DCC on growing cultures of *B. cepacia* 2a. Cells growing in 4-HB-salts medium (19 ml) were treated with 2,4-DCP or 3,5-DCC (1 ml) at various concentrations, and the effect on $OD_{540\ nm}$ was recorded. Rates of growth, expressed as percentages of the control, in the presence of 2,4-DCP (●); 3,5-DCC (◯)

The MIC for 2,4-DCP corresponds with the concentration at which induction of degradative activity for the phenol was inhibited. Uptake by 4-HB-grown cells from 0.5 mM 2,4-DCP, measured after removal of the cells by rapid Millipore filtration, by determination of the phenol remaining in the filtrate, was complete

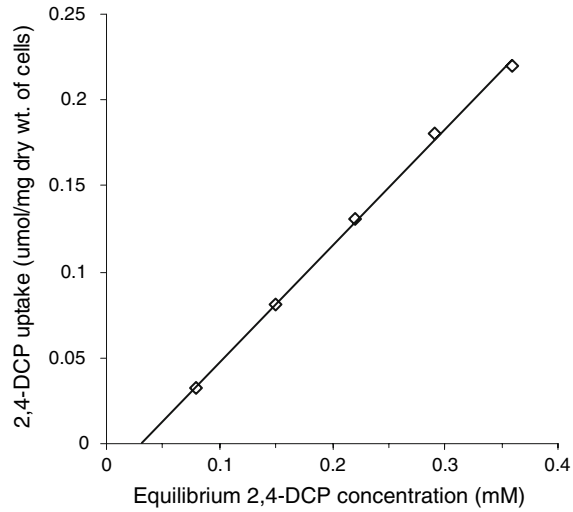

Fig. 4 Uptake isotherm for 2,4-DCP by suspensions of 4-HB-grown cells of *B. cepacia* 2a. 2,4-DCP (5.0 ml portions) yielding final concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mM were added to 20 ml of 4-HB-grown cells (0.79 mg dry weight ml^{-1}) in 50 mM Tris-DMG buffer, pH 6.5. After 5 min, samples were withdrawn, cells were centrifuged off, and the equilibrium 2,4-DCP concentration was measured in the supernatants. Only the proximal region of the isotherm is shown

within 5 min. Accordingly, suspensions were treated with concentrations up to 2.0 mM for 5 min, after which the cells were centrifuged off and the supernatants were assayed for remaining phenol. The data yielded a linear isotherm (Fig. 4) over the full range of phenol concentrations which extrapolated back to the abscissa at ∼0.03 mM 2,4-DCP. The uptake at 0.4 mM 2,4-DCP, at which growth ceased, was 0.18 μmol (mg dry weight of cells)$^{-1}$.

Addition of 2,4-DCP to 4-HB-grown cells caused extensive efflux of K^+ within 5 min, indicating cell membrane damage even at 0.1 mM 2,4-DCP, and was complete within 20 min (Fig. 5). Maximal loss (0.06 μmol K^+ (mg dry weight of cells)$^{-1}$; 43% of the exchangeable potassium detected after disrupting untreated cells) occurred with 0.3 mM 2,4-DCP; less leakage was observed with 0.5 mM 2,4-DCP. Loss of 260 nm-absorbing material, measured after 6 h exposure to 2,4-DCP (Fig. 6a), increased in proportion to the concentration applied, but significant loss was also seen with untreated cells. Efflux of P_i (Fig. 6b) began with 0.2 mM 2,4-DCP after ∼4 h exposure, becoming instant at 0.3 mM, but ceasing at 0.5 mM after 2 h exposure. The release of ribose-containing compounds (presumptive nucleotide

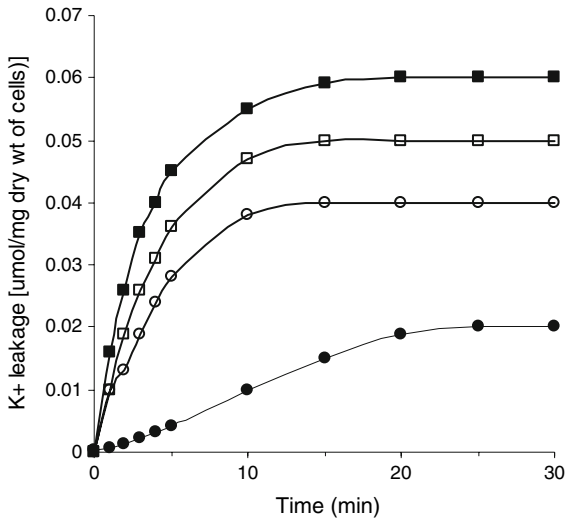

Fig. 5 Leakage of K^+ from suspensions of 4-HB-grown cells of *B. cepacia* 2a. 2,4-DCP solution (2.5 ml) was added to 10 ml of 4-HB-grown cells (0.62 mg dry weight ml^{-1}) suspended in 50 mM Tris-DMG buffer, pH 7.0. Leakage of K^+ was measured *in situ* using a valinomycin potassium ion-sensitive electrode, and is expressed as µmol K^+ (mg dry weight of cells)$^{-1}$. 2,4-DCP concentrations (mM): □ , 0.5; ■, 0.3; ◯, 0.1; ●, no addition

cofactors and possibly RNA) became apparent at 0.3 mM-2,4-DCP (Fig. 6b).

Chemostat studies

The toxicity of 2,4-DCP and 3,5-DCC prevented batch growth of *B. cepacia* 2a in significant concentrations of these compounds. In log phase growth on 2,4-D, strain 2a gave $t_D = 7$ h ($\mu_{max} = 0.10\ h^{-1}$), compared with 1.4 h for growth on succinate ($\mu_{max} = 0.49\ h^{-1}$). Chemostat cultures on limiting concentrations of these substrates were established at $D = 0.055\ h^{-1}$. Cultures on 2.26 mM 2,4-D were depleted of detectable carbon source, and a three-fold increase in 2,4-D concentration produced a corresponding increase in cell density, confirming carbon limitation. These growth conditions were adopted without variation for other carbon sources (Table 1).

Cultures established on succinate or glutamate remained stable when transferred to medium containing 2,4-DCP, though cell density was noticeably lower than on 2,4-D (Table 1). Substrate was utilized completely at dilution rates of up to 0.06 h^{-1}, but washout occurred at 0.08 h^{-1}. Attempts to obtain stable growth on 3,5-DCC alone, even with $D = 0.02\ h^{-1}$ failed; but cultures were successfully transferred at $D = 0.055\ h^{-1}$ from growth on succinate alone to media containing 3,5-DCC supplemented with succinate (Table 1), although cells were discoloured by catechol oxidation products.

Cellular and enzyme activities of chemostat-grown cells

At the time of this investigation, cell-free activity with 2,4-D was recoverable from 2,4-D-grown cells but was not accurately quantifiable, because the requirement for the co-substrate α-KG had not been identified, and the level of activity observed was therefore dependent on the varying amount of α-KG present in the cell crush. The same problem confronted Pieper et al. (1988); consequently measurements of 2,4-D consumption were conducted using whole cells. Succinate-grown cells oxidised 2,4-DCP after a lag (Table 2; Fig. 7a), and demonstrated enhanced oxygen uptake in the presence of 2,4-D and 3,5-DCC without utilising them; behaviour consistent with respiratory uncoupling, as noted also in *Pseudomonas putida* KT2440 (Benndorf et al. 2006).

Whole cells grown on any of the aromatic substrates consumed 2,4-D and 2,4-DCP without lag, with oxygen uptake lasting until their disappearance (Figs. 7b–d), but did not utilise 3,5-DCC (Figs. 7b–d). Cells washed in phosphate buffer were usually active towards 3,5-DCC, therefore the lack of response following washing with DMG may be an effect of buffer species.

Succinate-grown cells contained no 2,4-DCP hydroxylase or 3,5-DCC dioxygenase, but both enzymes were present in cells grown on the aromatic substrates (Table 2); and for cells supplied with 3,5-DCC and succinate, an increase in the ratio gave a corresponding increase in activity towards all three substrates.

Discussion

In common with other organisms (*e.g. Pseudomonas* NCIMB 9340, *C. necator* JMP 134), the dissimilative pathway for 2,4-D in strain 2a is inducible and proceeds *via* 2,4-DCP and *ortho*-cleavage of 3,5-DCC

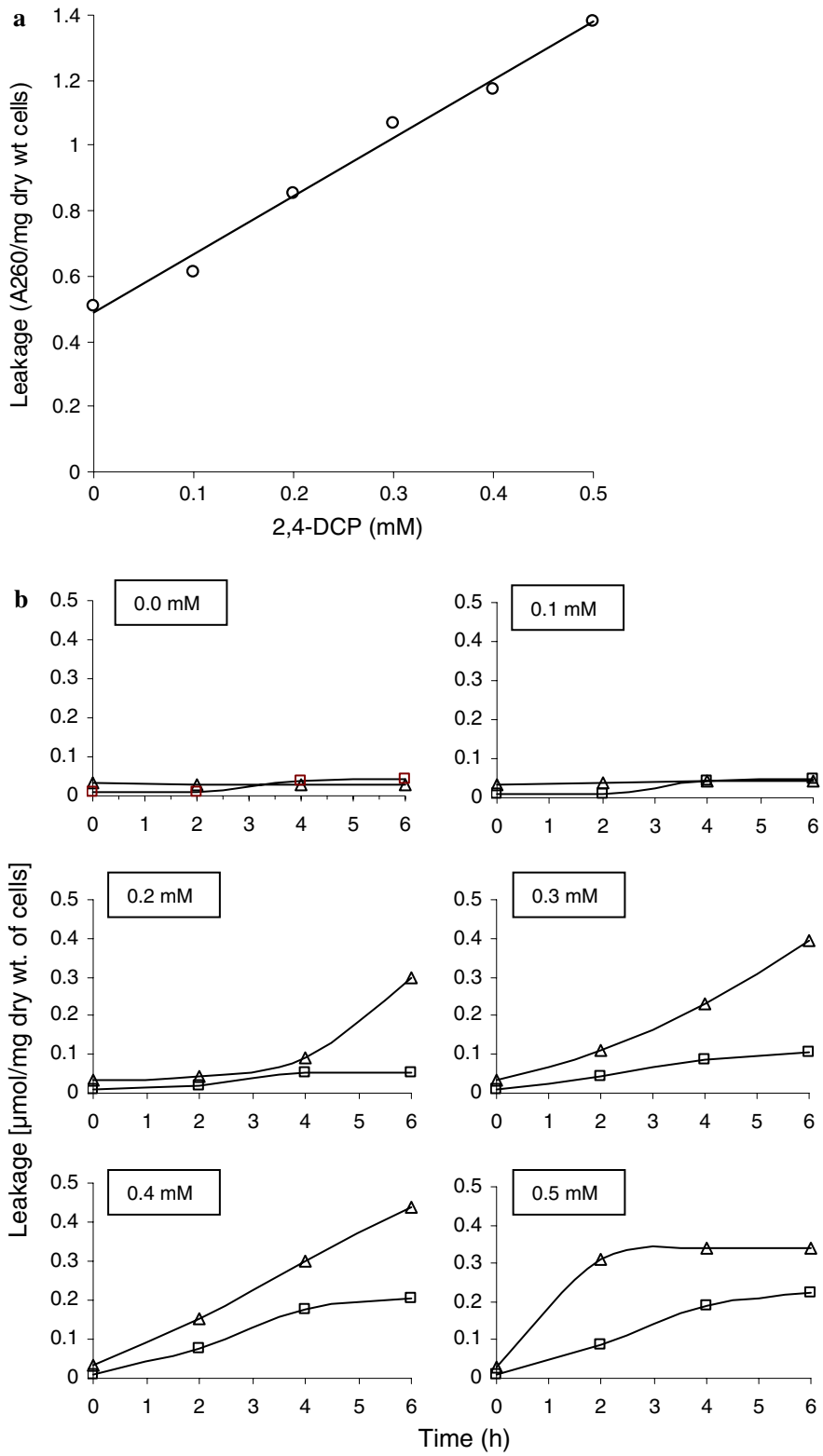

Fig. 6 Leakage of 260 nm-absorbing material, ribose and P_i from suspensions of 4-HB-grown cells of *B. cepacia* 2a. 2,4-DCP solution (5.0 ml) was added to 20 ml of 4-HB-grown cells (0.79 mg dry weight ml^{-1}) in 50 mM Tris-DMG buffer, pH 6.5. At intervals, samples were withdrawn, cells were removed by centrifugation and supernatants were assayed for ribose, P_i, or 260 nm-absorbing material. (**a**) Leakage of 260 nm-absorbing material expressed as $A_{260\ nm}$ (mg dry weight of cells)$^{-1}$, after extraction with ether (2 × 5 ml); (**b**) leakage of ribose and P_i, expressed as µmol (mg dry weight of cells)$^{-1}$ in the presence of the 2,4-DCP concentrations indicated: Δ, P_i; □, ribose equivalents

Table 1 Growth of *B. cepacia* 2a cells on various carbon sources

C source	Concentration (mM)	Population density (organisms ml^{-1})	Biomass concentration (mg dry wt l^{-1})	No of organisms (μg dry wt)$^{-1}$	Y^{a}_{app}
2,4-D	2.26	9.0×10^8	115	7.8×10^6	0.23
2,4-DCP	3.01	4.6×10^8	61	7.5×10^6	0.12
3,5-DCC + Succinate	1.21 + 2.71	1.2×10^9	ND	ND	NA
3,5-DCC + Succinate	0.60 + 3.62	1.1×10^9	124[b]	8.9×10^6	NA
Succinate	4.52	1.8×10^9	179[b]	1.0×10^7	0.34

The elemental carbon content of all media was 18.1 mm. Cell counts and dry weight determinations were performed as described in Methods

[a] Y_{app}, the apparent growth yield coefficient (g of cells formed (g of substrate consumed)$^{-1}$), is calculated on the assumption that all the substrate has been consumed

[b] Inferred from a plot of $OD_{540\ nm}$ readings versus dry weight of washed cells

ND, Not determined

NA, Not applicable

Table 2 Activities of *B. cepacia* 2a cells grown on various carbon sources

Growth medium		2,4-D consumption[a]		Enzyme activities	
C source	Concentration (mM)	nmol 2,4-D min^{-1}	μl O_2 min^{-1}	2,4-DCP hydroxylase[b]	3,5-DCC dioxygenase[c]
2,4-D	2.26	22.6	3.9	109	26.5
2,4-DCP	3.01	21.8	3.6	119	65.0
3,5-DCC + Succinate	1.21 + 2.71	ND	ND	76	13.3
3,5-DCC + Succinate	0.60 + 3.62	9.1	0.83	37	8.1
3,5-DCC + Succinate	0.30 + 4.07	6.2	ND	ND	ND
Succinate	4.52	<1	0.0	<1	<1

The elemental carbon content of all media was 18.1 mm. Washed cell and cell extract preparation, and enzyme assays were performed as described in Methods.

[a] Consumption is expressed on a *per mg dry weight of cells* basis, and oxygen uptake is corrected for endogenous respiration.

[b] Activity is expressed as nmol NADPH consumed min^{-1} (mg protein)$^{-1}$, after correcting for NADPH oxidase activity.

[c] Activity is expressed as nmol O_2 consumed min^{-1} (mg protein)$^{-1}$, after correcting for endogenous oxygen consumption.

ND, Not determined

by a Type II dioxygenase. The inability of strain 2a to transfer from nutrient agar to 2,4-D-salts unless first passaged on succinate-salts agar is puzzling, but could be due either to the difficulty of inducing a large number of biosynthetic pathways while being provided only with a potentially toxic substrate; or to some aspect of the metabolic control of the 2,4-D pathway perhaps involving the *lrp* gene. Cases and de

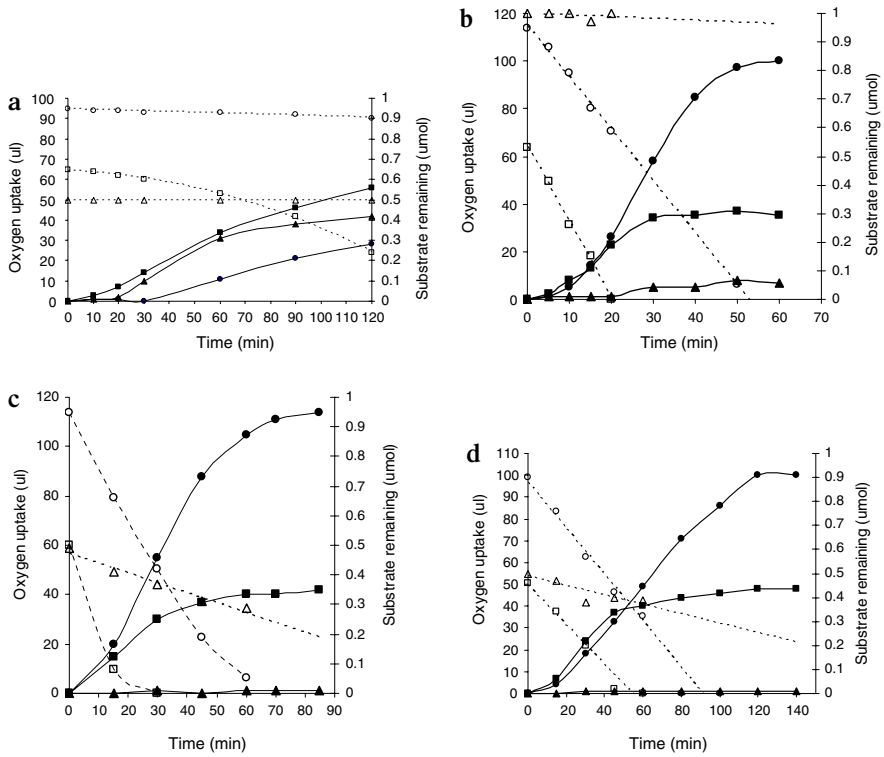

Fig. 7 Substrate utilization and oxygen uptake by suspensions of *B. cepacia* 2a cells grown on succinate, 2,4-D, 2,4-DCP or 3,5-DCC + succinate. Substrate consumption. Flasks contained 7.0 mg dry weight of cells in 50 mM Tris-DMG buffer, pH 6.5 (27 ml). Substrate solution or water (1.0 ml) was added to initiate reactions. At timed intervals, samples were withdrawn and acidified; cells were centrifuged off, and supernatants were assayed for remaining substrate. ◯, 2,4-D; □, 2,4-DCP; Δ, 3,5-DCC. Cells were supplied with the following quantities of the respective substrate (μmol) as follows: for cells grown on (**a**) succinate, 10, 6.7 and 5: (**b**) 2,4-D, 10, 6.3 and 10: (**c**) 2,4-DCP, 10, 5 and 5: (**d**) 3,5-DCC (0.60 mM) + succinate (3.62 mM), 10, 5 and 5. Some uptake curves are extrapolated to the times of the final measurements of oxygen consumption. Oxygen consumption. Uptakes were determined by conventional Warburg respirometry, and were corrected for endogenous respiration. Flasks contained in the main well cells (0.70 mg dry weight) in 2.7 ml of 50 mM Tris-DMG buffer, pH 6.5. Substrate solution or water (0.1 ml) was tipped from the sidearm to initiate reaction. ●, 2,4-D; ■, 2,4-DCP; ▲, 3,5-DCC. The amounts supplied were one-tenth those in the corresponding incubation mixtures described above, except in (**a**); the quantity of 2,4-DCP tipped was 1 μmol

Lorenzo (2001) observe that pathways assembled by organisms for the degradation of xenobiotic substances (such as, supposedly, 2,4-D) may use control mechanisms originally designed for other purposes.

MICs and MBCs of 2,4-DCP and 3,5-DCC were in accord with the concentrations causing complete growth inhibition in organisms during exponential growth in 4-HB-salts (0.4 mM and 0.2 mM respectively). Concentrations causing a 50% depression in the growth rate were similar to those recorded for the 2,4-D non-utiliser *Pseudomonas putida* KT2440 (Benndorf et al. 2006), but somewhat lower than values for the utiliser *D. acidovorans* MC1 (Benndorf and Babel 2002).

The isotherm for 2,4-DCP uptake by non-induced cells approximated to type S (cooperative uptake) in the classification system of Giles et al. (1960), characteristic of the surface adsorption of molecules oriented vertically, and with strong side-by-side association. Bean and Das (1966) described the uptake of several phenols by *Escherichia coli* as conforming to Type S. An initial low rate of uptake with respect to increase in phenol concentration terminated with a marked increase in the uptake rate, coinciding with the concentration at which 84% of the cells were killed within 1 min, and, making reasonable assumptions concerning cell size, the end of the initial phase of, for example, the uptake of

3-methyl-4-chlorocresol, estimating its surface area as 30Å^2 after consideration of the area occupied by 4-alkyl phenol (Giles et al. 1960), corresponded with the adsorption of an approximate 4-fold excess in the number of molecules required to form a surface monolayer, implying the onset of penetration into the cell. With strain 2a, adsorption of 2,4-DCP increased slowly with respect to concentration, to a value below 0.1 mM (not precisely defined by the present data), but thereafter uptake became easier due to the stabilisation offered by intermolecular attraction. Given a cell size of 1.0 × 0.5 μm, a cell surface area of 1.57 μm^2, and a surface area for 2,4-DCP of 30Å^2, then 5.2 × 10^6 molecules would be required to form a monolayer on each cell; therefore at 0.1 mM 2,4-DCP, the uptake of 0.032 μmol (2.02 × 10^{16} molecules) per mg dry weight (6.3 × 10^9 cells) would potentially cover only 39% of the cell surface. Unlike *E. coli*, the synergistic uptake of phenol appears to begin before the cell wall is covered, possibly due to a more lipophilic surface. This contrasts with the behaviour of *B. cepacia* in antibiotic uptake, in which the outer membrane shows only 11% of the permeability of that of *E. coli* to antimicrobial agents (Hancock 1998). The uptake of 0.18 μmol/mg, corresponding with the point at which growth ceased, was 2.2-fold in excess of the monolayer, implying surface penetration by the phenol. The sparing uptake by strain 2a at low 2,4-DCP concentrations is echoed in Fig. 3, where an increase in 2,4-DCP concentration initially only gradually increased the inhibition of cell growth rate. The isotherm gave no evidence of saturation even at 2 mM 2,4-DCP, possibly because strain 2a produces poly-β-hydroxybutyrate inclusions, generated even under carbon-limiting conditions, which probably absorb 2,4-DCP.

The effect of increasing 2,4-DCP concentration was to cause the release of intracellular components in ascending order of molecular size, consistent with progressive membrane damage (Lambert and Smith 1976a, b). At 0.1 mM 2,4-DCP, leakage of K^+, necessary for protein synthesis and respiration, was instant and ceased within 10 min, hence damage was more severe than necessary to cause simply respiratory uncoupling, since K^+ is larger than H^+. At 0.2 mM, P_i leakage began after ~4 h (Fig. 6), due to either slowly-progressing membrane damage permitting leakage of the larger ions, or activation of a nucleotide phosphatase such as ATPase; or to consumption of ATP in biosynthesis without regeneration. Loss of P_i would in any case diminish the possibility of resynthesising ATP. The onset at 0.3 mM 2,4-DCP of ribose leakage (nucleoside di- and triphosphates such as ATP; indicated also by loss of 260 nm-absorbing material), signalled further damage, and was accompanied by immediate loss of P_i. Above this point enzyme induction by 2,4-DCP was effectively halted (Fig. 2), probably due in part to quantitative efflux of ATP and other cofactors, and intermediary metabolites. Benndorf et al. (2006) ascribed diminished intracellular ATP levels in *P. putida* KT2440 to a lower rate of synthesis in the presence of 2,4-DCP, but such effects could also be caused by loss from leakage. The diminished K^+ efflux, and sudden cessation after 2 h of P_i leakage at 0.5 mM 2,4-DCP may indicate that greater phenol concentrations precipitated cytoplasmic protein or sealed the membrane and restricted the loss (Hugo 1976).

Cells grown in or treated with 3,5-DCC acquired a pink-brown appearance, becoming tanned by enzyme-assisted oxidation of the catechol to the corresponding quinone followed by polymerisation. Growth on 3,5-DCC in the absence of a more readily-utilised carbon source (here succinate) did not appear to be possible due to this apparently "suicidal" activity. Few attempts appear to have been made at prolonged culture of organisms on 3,5-DCC, perhaps because it also easily auto-oxidises, particularly above pH 7, where it is 12% ionised, which facilitates the process. At pH 6.5, where dissociation is only 4.1%, oxidation would be diminished, and the quinone could be back-reduced by reducing equivalents supplied by succinate metabolism. Succinate could possibly be dispensed with by reducing the oxygen supply. Benndorf et al. (2006) found that 3,5-DCC was much more toxic towards *P. putida* KT2440 than 2,4-D or 2,4-DCP, and actively uncoupled oxidative phosphorylation, as has also been found in *E. coli* (Schweigert et al. 2001).

Growth of strain 2a in a chemostat permitted approximate estimations of apparent growth yield coefficients (Y_{app}; Table 1). The value for 2,4-D was 0.23, somewhat larger the value reported for *C. eutrophus* JMP134 (0.15; Pieper et al. 1988). Y_{app} for 2,4-DCP was poor in comparison, perhaps due to uncoupling of oxidative phosphorylation, as seen in plant mitochondria (Gaur and Beevers 1959) and other bacteria (Benndorf et al. 2006). More probably, some of the phenol was purged from solution by the

air-stream and thereby rendered unavailable for assimilation. 2,4-DCP-grown cells also tended to take up more oxygen with both 2,4-D and 2,4-DCP than 2,4-D-grown cells, and oxygen uptake by succinate-grown cells occurred on all three aromatic substrates without stoichiometric substrate utilisation (Fig. 7d).

Organisms containing plasmid pJP4 are also active towards 2,4-D when grown on or presented with 2,4-DCP or 4-chlorocatechol (Kaphammer et al. 1990a; Kaphammer and Olsen 1990). Using a genetic approach, Filer and Harker (1997) identified the inducing agent for pJP4 as *cis, cis*-2,4-DCM. Assuming that only one compound is implicated in strain 2a, likely candidates are 3,5-DCC itself or a later metabolite such as *cis, cis*-2,4-DCM. Expression control in pJP4 involves at least two regulatory genes, *tfdR* and *tfdS* (Kaphammer et al. 1990; Kaphammer and Olsen 1990), but the absence of a gene corresponding with *tfdS* in strain 2a and the possession of *lrp* (Poh et al. 2002) has justified investigation of the adaptive responses made by this organism during growth on 2,4-D and its metabolic intermediates.

The identification of a post-ring fission metabolite as a probable inducer might explain the environmental persistence of the herbicide 2,4,5-trichlorophenoxyacetate (2,4,5-T; Audus 1964). Despite serving as a substrate to 2,4-D/α-KG dioxygenase (Fukumori and Hausinger 1993a, b; Poh et al. 2001), 2,4,5-T was utilised by none of the *Cupriavidus* strains described by Pemberton et al. (1979), and the product, 2,4,5-trichlorophenol is only slowly hydroxylated (Liu and Chapman 1984): strain 2a does not hydroxylate 2,4,5-trichlorophenol at all (Beadle and Smith 1982). The resistance of 2,4,5-T to attack could in part be ascribed to "microbial fallibility" (Alexander 1965); a failure of potential utilisers to form an inducer in the lower pathway.

References

Alexander M (1965) Biodegradation: problems of molecular recalcitrance and microbial fallibility. Adv Appl Microbiol 7:35–80

Audus LJ (1964) Herbicide behaviour in the soil. In: Audus LJ (ed) The physiology and biochemistry of herbicides. Academic Press, Oxford, pp 163–206

Beadle CA, Smith ARW (1982) The purification and properties of 2,4-dichlorophenol hydroxylase from a strain of *Acinetobacter* species. Eur J Biochem 123:323–332

Beadle CA, Kyprianou P, Smith ARW, Weight ML, Yon RJ (1984) Rapid purification of 2,4-dichlorophenol hydroxylase by biospecific desorption from 10-carboxydecylamino-Sepharose. Biochem Int 9:587–593

Bean HS, Das A (1966) The absorption by *Escherichia coli* of phenols and their bactericidal activity. J Pharm Pharmacol 18:107S–113S

Benndorf D, Babel W (2002) Assimilatory detoxification of herbicides by *Delftia acidovorans* MC1: induction of two chlorocatechol 1,2-dioxygenases as a response to chemostress. Microbiology 148:2883–2888

Benndorf D, Thiersch M, Loffhagen N, Kunath C, Harms H (2006) *Pseudomonas putida* KT2440 responds specifically to chlorophenoxy herbicides and their initial metabolites. Proteomics 6:3319–3329

Bollag J-M, Briggs GG, Dawson JE, Alexander M (1968a) 2,4-D metabolism: enzymatic degradation of chlorocatechols. J Agric Food Chem 16:829–833

Bollag J-M, Helling CS, Alexander M (1968b) 2,4-D metabolism: enzymatic hydroxylation of chlorinated phenols. J Agric Food Chem 16:826–828

Carrington B (1994) The biochemistry of 2,4-D dissimilation by Pseudomonas cepacia. PhD thesis, University of Greenwich, London

Cases I, de Lorenzo V (2001) The black cat/white cat principle of signal integration in bacterial promoters. EMBO J 20:1–11

Cooper CM, Fernstrom GA, Miller SA (1944) Performance of agitated gas-liquid contactors. Ind Eng Chem 36:504–509

Dawson RMC, Elliott EC, Elliott WH, Jones KM (1969) Data for Biochemical Research. 2nd edn. University Press, Oxford

Don RH, Pemberton JM (1981) Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J Bacteriol 145:681–686

Don RH, Weightman AJ, Knackmuss H-J, Timmis KN (1985) Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). J Bacteriol 161:85–90

Dryer RL, Tammes AR, Routh JI (1957) Determination of phosphorus and phosphatase with N-phenyl-*p*-phenylenediamine. J Biol Chem 225:177–183

Evans WC, Smith BSW, Fernley HN, Davies JI (1971a) Bacterial metabolism of 2,4-dichlorophenoxyacetate. Biochem J 122:543–551

Evans WC, Smith BSW, Moss P, Fernley HN (1971b) Bacterial metabolism of 4-chlorophenoxyacetate. Biochem J 122:509–517

Filer K, Harker AR (1997) Identification of the inducing agent of the 2,4-dichlorophenoxyacetic acid pathway encoded by plasmid pJP4. Appl Environ Microbiol 63:317–320

Fisher PR, Appleton J, Pemberton JM (1978) Isolation and characterisation of the pesticide-degrading plasmid pJP1 from *Alcaligenes paradoxus*. J Bacteriol 135:798–804

Fukumori F, Hausinger RP (1993a) *Alcaligenes eutrophus* JMP134 2,4-dichlorophenoxyacetate monooxygenase is an α-ketoglutarate-dependent dioxygenase. J Bacteriol 175:2083–2086

Fukumori F, Hausinger RP (1993b) Purification and characterisation of 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenase. J Biol Chem 268:24311–24317

Gaunt JK, Evans WC (1971a) Metabolism of 4-chloro-2-methylphenoxyacetate by a soil pseudomonad: preliminary evidence for the metabolic pathway. Biochem J 122:519–526

Gaunt JK, Evans WC (1971b) Metabolism of 4-chloro-2-methylphenoxyacetate by a soil pseudomonad: ring-fission, lactonizing and delactonizing enzymes. Biochem J 122:533–542

Gaur BK, Beevers H (1959) Respiratory and associated responses of carrot disks to substituted phenols. Plant Physiol 34:427–432

Giles CH, MacEwan TH, Nakhwa SN, Smith D (1960) Studies in adsorption. XI. A system of classification of solution adsorption isotherms, and its use in the diagnosis of adsorption mechanisms and in measurements of specific surface areas of solids. J Chem Soc 3973–3993

Hancock REW (1998) Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative Gram-negative bacteria. Clin Infect Dis 27(Suppl 1):S93-S99

Herbert D, Phipps PJ, Strange RE (1971) Chemical analysis of microbial cells. In: Norris JR, Ribbons DW (eds) Methods in Microbiology, vol 5B. Academic Press, London & New York, pp 209–344

Hugo WB (1976) Survival of microbes exposed to chemical stress. In: Gray TRG, Postgate JR (eds) The survival of vegetative microbes. 26th Symp Soc Gen Microbiol. University Press, Cambridge, pp 383–413

Kaphammer B, Kukor JJ, Olsen RH (1990) Regulation of *tfdCDEF* by *tfdR* of the 2,4-dichlorophenoxyacetic acid degradation plasmid pJP4. J Bacteriol 172:2280–2286

Kaphammer B, Olsen RH (1990) Cloning and characterisation of *tfdS*, the repressor-activator gene of *tfdB*, from the 2,4-dichlorophenoxyacetic acid catabolic plasmid pJP4. J Bacteriol 172:5856–5862

Lacoste RJ, Venable SH, Stone JC (1959) Modified 4-amino-antipyrine colorimetric method for phenols. Application to an acrylic monomer. Anal Chem 31:1246–1249

Lambert PA, Hammond SM (1973) Potassium fluxes, first indications of membrane damage in micro-organisms. Biochem Biophys Res Comm 54:796–799

Lambert PA, Smith ARW (1976a) Antimicrobial action of dodecyldiethanolamine: induced membrane damage in *Escherichia coli*. Microbios 15:191–202

Lambert PA, Smith ARW (1976b) Antimicrobial action of dodecyldiethanolamine: activation of ribonuclease I in *Escherichia coli*. Microbios 17:35–49

Liu T, Chapman PJ (1984) Purification and properties of a plasmid-encoded 2,4-dichlorophenol hydroxylase. FEBS Letters 173:314–318

Mitchell AC (1924) Osmium tetroxide as a reagent for the estimation of tannins and their derivatives. Analyst 49:162–169

Pemberton JM, Corney B, Don RH (1979) Evolution and spread of pesticide-degrading ability among soil organisms. In: Kimmis KN, Puhler A (eds) Plasmids of Medical, Environmental and Commercial Importance. Elsevier/North Holland Biomedical Press, pp 287–299

Pieper DH, Reineke W, Engesser K-H, Knackmuss H-J (1988) Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP134. Arch Microbiol 150:95–102

Pirt SJ (1975) Principles of microbe and cell cultivation. Blackwell, Oxford, p 37

Poh RP, Smith ARW, Bruce IJ (2002) Complete characterisation of Tn*5530* from *Burkholderia cepacia* strain 2a (pIJB1) and studies of 2,4-dichlorophenoxyacetate uptake by the organism. Plasmid 48:1–12

Poh R, Xia X, Bruce IJ, Smith ARW (2001) 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenases from *Burkholderia cepacia* 2a and *Ralstonia eutropha* JMP134. Microbios 105:43–63

Schlömann M (1994) Evolution of chlorocatechol catabolic pathways: conclusions to be drawn from comparisons of lactone hydrolases. Biodegradation 5:301–321

Schweigert N, Hunziker RW, Escher BI, Eggen RIK (2001) Acute toxicity of (chloro-)catechols and (chloro-)catechol-copper combinations in *Escherichia coli* corresponds to their membrane toxicity in vitro. Environ Toxicol Chem 20:239–247

Sebastianelli A, Bruce IJ (2007) Tn*5530* from *Burkholderia cepacia* strain 2a encodes a chloride channel protein essential for the catabolism of 2,4-dichlorophenoxyacetic acid. Environ Microbiol 9:256–265

Sharpee KW, Duxbury JM, Alexander M (1973) 2,4-Dichlorophenoxyacetate metabolism by *Arthrobacter* sp.: accumulation of a chlorobutenolide. Appl Microbiol 26: 445–447

Streber WG, Timmis KN, Zenk MH (1987) Analysis, cloning and high-level expression of 2,4-dichlorophenoxyacetate monooxygenase gene *tfdA* of *Alcaligenes eutrophus* JMP134. J Bacteriol 169:2950–2955

Tiedje JM, Alexander M (1969) Enzymatic cleavage of the ether bond of 2,4-dichlorophenoxyacetate. J Agric Food Chem 17:1080–1084

Tiedje JM, Duxbury JM, Alexander M, Dawson JE (1969) 2,4-D metabolism: pathway of degradation of chlorocatechols by *Arthrobacter* sp. J Agric Food Chem 17:1021–1026

Umbreit WW, Burris RH, Stauffer JF (1964) Manometric Techniques, 4th edn. Burgess, Minneapolis, p 157