Flux limitations in the *ortho* pathway of benzoate degradation of *Alcaligenes eutrophus*: metabolite overflow and induction of the *meta* pathway at high substrate concentrations

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The growth behaviour of *Alcaligenes eutrophus* using various concentrations of benzoate was investigated. In batch culture, growth was exponential and growth rate (μ) and yields (Y) were high [$\mu = 0.51 \text{ h}^{-1}$ and $Y_{X/\text{benzoate}} = 0.56 \text{ mol carbon (mol carbon)}^{-1}$] when low concentrations of benzoate ($< 5 \text{ mM}$) were used. These kinetic parameters were close to the maxima determined in a benzoate-limited chemostat [$\mu_{\text{max}} = 0.55 \text{ h}^{-1}$ and $Y_{X/\text{benzoate}}^{\text{max}} = 0.57 \text{ mol carbon (mol carbon)}^{-1}$] and the part of the energy for maintenance was limited ($m_{\text{ATP}} = 4.3 \pm 2.2 \text{ mmol ATP g}^{-1} \text{ h}^{-1}$). When higher concentrations of benzoate were used (up to 40 mM), several metabolic limitations appeared. The specific rate of benzoate consumption was not altered, whereas growth was inhibited [$K_i(\text{benzoate}) \approx 27 \text{ mM}$]. Furthermore, high concentrations of catechol together with some 1,2-dihydro-1,2-dihydroxybenzoate (DHB) transiently accumulated in the medium. The accumulation of catechol was attributed to limiting flux through catechol 1,2-dioxygenase estimated to be $5.2 \text{ mmol g}^{-1} \text{ h}^{-1}$, whereas that of DHB was provoked by an imbalance in the NADH/NAD⁺ intracellular content. The direct consequence of DHB accumulation was the induction of the *meta* pathway for the degradation of catechol, and this pathway contributed up to 20% of the total flux of catechol to the central metabolism. Finally, when very high concentrations of benzoate were used (55 mM), both growth and the specific rate of benzoate degradation were diminished due to a strong decrease in benzoate 1,2-dioxygenase specific activity.

Keywords: *Alcaligenes eutrophus*, benzoate, *meta*-cleavage pathway, *ortho*-cleavage pathway, regulation

INTRODUCTION

Alcaligenes eutrophus, a commonly occurring soil bacterium, possesses chromosome-encoded pathways for the degradation of aromatic compounds (Johnson & Stanier, 1971a). In addition, plasmids extend the range of substrates degraded to pollutants such as 2,4-dichlorophenoxy-acetate (Rasul-Chaudhry & Chapalmadugu, 1991), chlorobenzenes (Don *et al.*, 1985), methylaromatics

(Pieper *et al.*, 1985) or polychlorinated biphenyls (Bedard *et al.*, 1987; Springael *et al.*, 1993).

In *A. eutrophus*, the simple aromatic compound, benzoate, is metabolized via the *ortho* (also named β -ketoadipate) pathway (Fig. 1), which has received far less study than the plasmid-encoded *meta*-cleavage pathways. The highly complex biochemical regulation of the *ortho* pathway was partly elucidated for *A. eutrophus* strain 335 by Johnson & Stanier (1971b). Benzoate and *cis,cis*-muconate appeared to be key intermediates in the induction of the enzymes of the pathway (Johnson & Stanier, 1971b; Stanier & Ornston, 1973), but despite those early reports there remain gaps in the knowledge of the regulation, and to date only two genes of the pathway (*catD* and *pcaD*

Abbreviations: B12O, benzoate 1,2-dioxygenase; C12O, catechol 1,2-dioxygenase; C23O, catechol 2,3-dioxygenase; DHB(DH), 1,2-dihydro-1,2-dihydroxybenzoate (dehydrogenase); MCI, muconate cycloisomerase; PDH, pyruvate dehydrogenase.

Fig. 1. The *ortho* pathway (also named the β -ketoadipate pathway) for the degradation of benzoate in *A. eutrophus* (adapted from Johnson & Stanier, 1971a and Reiner & Hegeman, 1971).

encoding the two lactone hydrolases of *A. eutrophus*) have been cloned and studied (Schlömann *et al.*, 1991).

Despite the rapid degradation of aromatic compounds by *A. eutrophus* or *Pseudomonas* sp. observed in the laboratory, biodegradation appears to be far less efficient in natural environments. The presence of other compounds such as

organic acids has sometimes been implicated and possible catabolite repression effects have already been examined. However, repression of the *ortho* pathway by such compounds has not been unambiguously demonstrated and the molecular mechanisms remain obscure (Ornston, 1966; Duetz *et al.*, 1994; Holtel *et al.*, 1994; MacGregor *et al.*, 1992; Ampe & Lindley, 1995). Another possible cause for limitation of the catabolism of aromatic compounds by pseudomonads and related organisms would be oxygen limitation, a substrate in the *ortho*- and *meta*-cleavage pathways. This aspect, though not well studied, has already been evoked in the case of benzoate degradation by *A. eutrophus* (Dols *et al.*, 1994; Zhou & Crawford, 1995). However, most studies reported on the regulation of the catabolism of aromatic compounds have been performed with low or very low concentrations of substrates (generally below 5 mM) which do not necessarily reflect the stress conditions that bacteria encounter in on-site 'end-of-pipe' waste water treatment where high concentrations might be expected with possible toxic effects.

This paper relates the study of the degradation of benzoate, a model substrate for the *ortho* pathway, by *A. eutrophus*, with particular emphasis on the effect of substrate concentration. Firstly, a kinetic analysis of the growth of the organism on low concentrations of benzoate was performed. Then, the effects of high concentrations of benzoate on growth and biodegradation abilities, and the response of the cell to this stress were investigated.

METHODS

Bacterial strains. *Alcaligenes eutrophus* strain 335 (ATCC 17697) was obtained from LMG (Brussels, Belgium). *A. eutrophus* strain B9 lacking 1,2-dihydro-1,2-dihydroxybenzoate (DHB) dehydrogenase (DHBDH, Reiner & Hegeman, 1971) was kindly provided by George Hegeman (Indiana University, Bloomington, USA).

Medium. The mineral salts medium used for growth of *A. eutrophus* was derived from that described by Johnson & Stanier (1971a) and contained (l^{-1}): nitrilotriacetic acid (200 mg), $FeSO_4 \cdot 7H_2O$ (7 mg), $MgSO_4 \cdot 7H_2O$ (580 mg), $CaCl_2 \cdot 2H_2O$ (67 mg), NaCl (292 mg), $(NH_4)_2SO_4$ (2 g), $ZnSO_4 \cdot 7H_2O$ (10.95 mg), $MnSO_4 \cdot H_2O$ (1.54 mg), $CuSO_4$ (0.251 mg), $CoCl_2 \cdot 6H_2O$ (0.2 mg), H_3BO_4 (0.114 mg), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (2 mg) and $NiCl_2 \cdot 6H_2O$ (0.04 mg). The pH of the basal salt medium was adjusted to 7.4 and the medium was autoclaved. A stock solution of 1 M potassium phosphate (pH 7.4) was autoclaved separately and added to a final concentration of 40 mM. Carbon sources were filter-sterilized and added aseptically to the sterile salts medium.

Cultivation. One litre shake flasks with 150 ml medium were used for preliminary experiments on growth rates and benzoate consumption rates ($q_{benzoate}$). A 1.5 l bioreactor from Setric was used for all other experiments. The temperature was maintained at 30 °C, the pH at 7.4 with controlled addition of H_3PO_4 (1 M) and the oxygen partial pressure at 70% saturation (around 0.15 mM oxygen under these conditions) by agitation and air flow rate variation. The bioreactor was inoculated with 10% (v/v) late exponential phase inoculum grown in shake flasks with the same medium. Benzoate was used as sole carbon source for the inoculum. After inoculation, samples were periodically

withdrawn from the bioreactor with sterile syringes. Batch cultures were performed in triplicate.

For chemostat study, culture conditions were similar to those of batch culture except that the benzoate concentration in the inflowing medium was fixed at 20 mM. The dilution rates tested ranged from 0.1 to 0.5 h⁻¹.

Measurement of fermentation parameters. Biomass was measured by cell dry weight determination. A biomass formula of C₄H_{6.9}N_{0.98}O_{1.63} (with 5% ash) determined by elemental analysis was used for calculations. The concentrations of benzoate, DHB, catechol, *cis,cis*-muconate, acetate and hydroxybutyrate were analysed by HPLC (HP 1050, Hewlett Packard) equipped with an integrator (HP 3396A) and an automatic injector (SP 8775 from Spectra Physic France). Detection was made at 210 nm with an HP variable wavelength detector (Hewlett Packard, HP series 1050). The separation was obtained with an AminexR HPX-87H (Bio-Rad) column (300 × 7.8 mm) and the operating conditions were as follows: temperature, 65 °C; mobile phase, H₂SO₄ (5 mM)/CH₃CN (7% v/v); flow rate, 0.8 ml min⁻¹.

Determination of enzyme activities. Benzoate 1,2-dioxygenase (B12O) activity was estimated with whole cells directly sampled from the bioreactor, washed with 100 mM Tris/HCl (pH 7.5) and resuspended in Tris buffer. The cells were placed in a biological oxygen monitor (YSI 5300, Yellow Springs) with 3 ml of the same buffer containing 1 mM benzoate. Blanks without benzoate were prepared for each assay. B12O activity was expressed as mmol benzoate consumed (g cell dry wt)⁻¹ h⁻¹ and takes into consideration that 2 mol oxygen are consumed per mol benzoate (i.e. oxygen consumption rates are divided by two). This method was adapted from that described by Farr & Cain (1968). Phenol hydroxylase (phenol 2-monooxygenase, EC 1.14.13.7) was assayed using the same method with phenol (1 mM) replacing benzoate. For all other enzymes, cell-free extracts were prepared. Approximately 50–100 mg (wet wt) of freshly harvested cells were washed twice in 100 mM Tris/HCl (pH 7.5) at 4 °C and resuspended in 10 ml Tris/carballylate buffer (tricarballic acid, 9 mM; Tris/HCl, 35 mM; MgCl₂, 5 mM; glycerol, 20%, v/v; pH 7.8). The cells were disrupted by sonication and the resulting crude extracts were centrifuged to obtain soluble extracts which were used to assay enzyme activities. Enzymes of the *ortho* and *meta* pathways: DHBBDH (Reiner, 1971), catechol 1,2-dioxygenase (C12O, EC 1.13.11.1; Neidle & Ornston, 1990), *cis,cis*-muconate lactonizing enzyme or muconate cycloisomerase (MCI, EC 5.5.1.1; Meagher *et al.*, 1990) and catechol 2,3-dioxygenase (C23O, EC 1.13.11.2; Kataeva & Golovleva, 1990) were assayed by published methods except that the buffer was replaced by Tris/HCl (100 mM, pH 7.5). Blanks without substrate were prepared for each extract. Acetyl-CoA synthetase (acetate-CoA ligase, EC 6.2.1.1) activity was determined by the enzyme assay procedure of Oberlies *et al.* (1980) in which the formation of AMP from ATP is monitored by coupling the reaction to the oxidation of NADH via adenylate kinase, pyruvate kinase and lactate dehydrogenase. Blanks without CoA and ATP were prepared for each extract. Isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) activities were determined at pH 7.5 by the procedures described by Maloy *et al.* (1980). NADH oxidase was estimated using the procedure described by Mandelstam & Jacoby (1965). Protein was determined by the method of Lowry. Activities are expressed as nmol min⁻¹ (mg protein)⁻¹.

Kinetic measurements. Michaelis constants (K_m) for catechol with C12O and C23O were measured in crude extracts of cells grown in benzoate (5 mM) or phenol (5 mM), respectively,

under conditions in which co-induction of both enzymes could be excluded. The K_m for *cis,cis*-muconate of MCI was measured on crude extracts of cells grown in 5 mM benzoates. Constants were estimated with substrate at concentrations ranging from 0.6 to 10 times the respective values. The K_m values of both catechol dioxygenases for oxygen were estimated with initial oxygen contents ranging from 1 to 100% saturation with air at 30 °C (100% saturation corresponds to 0.22 mM oxygen under these conditions) in the oxygen monitor described above. The kinetic parameters were determined from double reciprocal plots.

Extraction and estimation of intracellular metabolite concentrations. The extraction procedure optimized by Le Bloas *et al.* (1993) was used. Cell samples of known dry weight were removed directly from the culture, frozen immediately in liquid nitrogen and stored at -80 °C. To avoid artefacts due to variable amounts of biomass during extraction procedures, the cell sample volume was adjusted (and supplemented with fresh medium) to obtain a final biomass concentration in each sample of 200 mg cell dry wt l⁻¹. While thawing in ice, 100 µl KOH (10 M) or 200 µl concentrated HCl were added to give a final pH of 12.5 or 1.2 for alkaline or acid extraction, respectively. In both cases quenching times were less than 0.5 s. The acid extraction procedure was achieved by incubating the HCl-treated sample (pH 1.2) at 50 °C for 8 min. before neutralizing with KOH (10 M) while agitating vigorously. After centrifugation at 12000 g for 10 min at 4 °C, the supernatant was used for assays. Acid-labile NADH was extracted by incubating the KOH-treated samples at room temperature for 10 min. After centrifugation at 12000 g for 10 min at 4 °C, the supernatant was immediately tested for NADH without neutralizing to avoid destruction of NADH. NAD⁺ and NADH were assayed using the enzyme procedures described by Le Bloas *et al.* (1993) by measuring the change of NADH fluorescence with a spectrofluorometer (Hitachi F2000).

Uncoupling experiments. The possible uncoupling effects of benzoate, DHB, catechol and acetate on respiratory activity were assayed as follows. Cells grown on fructose were sampled during exponential growth phase and placed in the oxygen monitor described above. Oxygen consumption was followed in the presence or absence of the compound tested.

Metabolic fluxes. Metabolic fluxes (carbon and energy) were calculated using the BIONET stoichiometric modelling approach (Vallino & Stephanopoulos, 1990), based on anabolic precursor (intermediary metabolites and coenzymes) requirements as cited for *Escherichia coli* (Ingraham *et al.*, 1983). This method assumes that the intermediary metabolite pools remain approximately constant, or at least undergo variations in concentrations which may be considered insignificant in comparison to the flux.

Chemicals. All chemicals were of analytical grade. *cis,cis*-Muconate was synthesized by the procedure of Elvidge *et al.* (1950). DHB was prepared using *A. entrophus* strain B9 by the procedure of Reiner (1971). Purified C12O from *A. entrophus* CH34 was a kind gift from G. Sauret-Ignazi (University Joseph Fourier, Grenoble, France). All other substrates, enzymes and coenzymes were obtained from Sigma.

RESULTS AND DISCUSSION

Growth with non-inhibitory concentrations of benzoate

When *A. entrophus* was grown in batch culture in media containing 4 mM benzoate with a further addition of 5 mM to prolong growth, kinetic parameters were

Fig. 2. Flux distribution within the central metabolic pathways based on the kinetic data from exponential growth in batch cultures with (a) low (< 5 mM) and (b) high (30 mM) concentrations of benzoate. The flux estimation relating to the culture with 30 mM benzoate used the specific rates measured after 5 h cultivation (see arrow in Fig. 4), a growth rate of 0.3 h^{-1} and a specific benzoate consumption rate of $5.2 \text{ mmol g}^{-1} \text{ h}^{-1}$.

constant $[\mu = 0.51 \pm 0.02 \text{ h}^{-1}; q_{\text{benzoate}} = 5.23 \pm 0.1 \text{ mmol g}^{-1} \text{ h}^{-1}; Y_{\text{X/benzoate}} = 0.56 \pm 0.02 \text{ mol carbon (mol carbon)}^{-1}; q_{\text{O}_2} = 19.3 \pm 2.1 \text{ mmol g}^{-1} \text{ h}^{-1}; v_{\text{CO}_2} = 18.8 \pm 2.8 \text{ mmol g}^{-1} \text{ h}^{-1}; \text{RQ} = 0.97 \pm 0.10]$ and a true exponential phase was observed. Using the data calculated for *E. coli* for the biosynthesis of building blocks (Ingraham *et al.*, 1983; Holms, 1986) together with the kinetic constants observed during exponential growth on benzoate, carbon flux distribution through the central metabolism and associated energetic yields were estimated (Fig. 2a; Table 1). These estimations assumed that benzoate degradation via the *ortho* pathway led to equimolar quantities of CO_2 , succinate and acetyl-CoA being formed. They indicate that little excess (or so-called 'maintenance') energy was produced during this exponential phase, and that the cells had no apparent requirement for pyruvate dehydrogenase (PDH) activity, though the physiological importance of this observation remains obscure. The PDH of *A. eutrophus* has recently been demonstrated to share a common subunit (dihydrolipoamide dehydrogenase) with other enzymes in the central metabolism (Hein & Steinbüchel, 1994), and the absence of PDH may allow the cell to use its pool of dihydrolipoamide dehydrogenase for other key reactions. To confirm the kinetic results seen in batch cultures, chemostat cultures of *A. eutrophus* were established with benzoate as the sole carbon source and limiting substrate.

Table 1. Energy balance based on energy fluxes calculated for batch culture growth on low concentrations (< 5 mM) of benzoate as depicted in Fig. 2a

	Co-enzyme production-catabolism ($\text{mmol g}^{-1} \text{ h}^{-1}$; P/O = 2)*	Co-enzyme consumption-anabolism ($\text{mmol g}^{-1} \text{ h}^{-1}$; P/O = 2)*
NADH_2	5.23	-1.81†
NADPH_2	8.88	9.29
FADH_2	8.0	0
ATP	3.4	19.42
Total	35.49 equiv. ATP	34.38 equiv. ATP

* P/O, efficiency of energy conservation within the respiratory chain (number of phosphorylation sites per atom oxygen consumed).

† The negative value represents an additional production of NADH_2 during the synthetic pathways.

Steady-states at dilution rates (D) ranging from 0.1 to 0.5 h^{-1} were established and the kinetic behaviour thus obtained was evaluated. Plotting q_{benzoate} versus μ gave a straight relationship whose slope gave a theoretical

Fig. 3. Specific growth rate (a) and B12O activity (b) as a function of benzoate concentration during batch culture in a bioreactor. Data are instantaneous growth rates collected from the start of cultures with 4 (∇), 17 ($\square$), 30 ($\bullet$), 40 ($\circ$) and 55 ($\blacksquare$) mM benzoate prior to the accumulation of products in the medium.

$Y_{X/benzoate}^{max}$ of 0.57 ± 0.02 mol carbon (mol carbon)⁻¹, and when extrapolated to $\mu = 0$, enabled a maintenance coefficient of $m = 0.22 \pm 0.11$ mmol benzoate g⁻¹ h⁻¹ (or 0.027 ± 0.014 g g⁻¹ h⁻¹) to be estimated. Increasing D to 0.6 h⁻¹ provoked the washout of the culture but enabled a μ_{max} value of 0.55 ± 0.02 h⁻¹ to be estimated.

The maintenance coefficient estimated here is of a similar magnitude to that observed for a variety of micro-organisms during growth on glucose (Pirt, 1975). It would appear that benzoate is a good carbon and energy source for the growth of *A. eutrophus* with the capacity to support both rapid growth and high yields of biomass formation.

Inhibition of growth on high concentrations of benzoate

When batch cultures of *A. eutrophus* were established in which different initial benzoate concentrations (17, 30, 40 and 55 mM) were used, the specific growth rate diminished as the initial benzoate concentration was increased

Fig. 4. Kinetics of growth and substrate consumption of *A. eutrophus* in batch culture on 30 mM benzoate. $\blacksquare$, benzoate; $\blacklozenge$, catechol; $\circ$, DHB; ∇ , acetate; $\bullet$, biomass; dashed line, $q_{benzoate}$; solid line, $q_{benzoate} - v_{catechol}$; dotted line, μ . A similar profile was observed for growth on 17 or 40 mM benzoate, except that for 40 mM benzoate, only a little catechol accumulated whereas up to 0.77 mM DHB were found in the medium at the end of the culture period.

(Fig. 3a). No true exponential phase could be observed in these cultures; growth rate increased as benzoate was degraded, i.e. as residual benzoate concentration decreased (Figs 4 and 6). The inhibition constant K_i at which $\mu = \mu_{max}/2$, was estimated to be 27 ± 3 mM. Such growth behaviour, depicted in Fig. 3(a), is typical of the growth inhibition by a weak acid. This phenomenon has already been described for *E. coli*, *Clostridium thermoaceticum* and other bacteria, especially through the inhibition of growth by the production of acetic acid (Wang & Wang, 1984; Luli & Strohl, 1990), though benzoic acid has also been reported to inhibit the growth of *E. coli* (Salmond *et al.*, 1984). These authors attributed the inhibitory effect of weak acids to a decrease in the capacity of the micro-organism to maintain pH homeostasis. The inhibitory effect exerted by benzoate is different from that described for the growth of *P. putida* with phenol (Hill & Robinson, 1975; Yang & Humphrey, 1975). This latter compound also inhibits growth, but the kinetics observed fit with Haldane's model (Haldane, 1965).

Table 2. Effect of benzoate and other compounds involved in the *ortho* pathway on the specific respiration rate of exponential phase fructose-grown cells of *A. eutrophus*

Compound tested	Concentration (mM)	Respiration with effector
		Respiration on fructose alone
Benzoate	10	0.98
	25	0.92
	50	0.66
	100	0.47
Catechol	1	0.86
	5	0.69
	10	0.38

The uncoupling of the proton-motive force has been postulated to be at the origin of growth inhibition by organic acids (Herrero *et al.*, 1985). Thus, the effect of benzoate and catechol (compounds seen to accumulate under conditions of growth on benzoate) on the respiration rate of fructose-grown cells was assayed. Results showed that neither of the compounds tested provoked an increase in the respiration rate of fructose-grown cells (Table 2). Indeed, both benzoate and catechol led to diminished rates of oxygen consumption while both DHB and acetate at concentrations several times higher than measured in the cultures had no effect on respiratory activity. It would appear that respiratory uncoupling cannot account for the inhibitory effect of benzoate.

Kinetic study of growth with 17–40 mM benzoate

Growth of *A. eutrophus* in batch cultures with benzoate concentrations of 17, 30 and 40 mM gave similar kinetic profiles, and only the culture with 30 mM will be described in detail (Fig. 4). In the first phase of growth, specific rates of benzoate (q_{benzoate}) degradation increased progressively to reach $7 \pm 0.3 \text{ mmol g}^{-1} \text{ h}^{-1}$. At values of q_{benzoate} in excess of that observed in batch cultures with non-inhibitory concentrations of benzoate (see Fig. 2a), catechol was seen to accumulate in the medium. Catechol concentration reached 3.7 mM in both cultures with 17 or 30 mM benzoate and was reconsumed in the later phase of the culture. The net flux through the *ortho* pathway was estimated by the difference between the specific rate of benzoate consumption and that of catechol accumulation (i.e. $q_{\text{benzoate}} - v_{\text{catechol}}$). This value was found to be constant and equal to $5.2 \pm 0.2 \text{ mmol g}^{-1} \text{ h}^{-1}$ (Fig. 4), i.e. the same as the q_{benzoate} found for non-inhibitory concentrations of benzoate. As well as the accumulation of catechol, small amounts of DHB were detected in the medium (maximum concentration around 0.6 mM). During the period of culture in which catechol was reconsumed, acetate accumulated together with trace amounts of hydroxybutyrate in the medium. This co-

Fig. 5. Specific activity and NADH/NAD⁺ ratio profiles throughout culture with 30 mM benzoate described in Fig. 4. ■, B12O; ●, C12O; ▽, MCI; ○, DHB; △, [NADH]/[NAD⁺]; □, C23O; ◆, NADH oxidase. Acetyl-CoA synthetase and phenol hydroxylase activities could not be detected at significant levels [i.e. activity measurements were always below 1 mU (mg protein)⁻¹ and 0.04 mmol g⁻¹ h⁻¹, respectively]. Vertical bars show the SD for the measurements based upon six repetitions for samples taken throughout a single culture. Similar values were obtained for three different batch cultures with 30 mM benzoate.

incided with the appearance of a bright yellow colouration of the supernatant, typical for the accumulation of 2-hydroxymuconic semialdehyde. The presence of this product of the *meta*-cleavage of catechol was confirmed by

Table 3. Induction of *ortho*- and *meta*-cleavage pathway enzymes by intermediates in the degradation of benzoate

Data are the means of four to six determinations $\pm$ SD. ND, not determined.

Substrate	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]			
	DHBDH	C12O	MCI	C23O
Benzoate	596 $\pm$ 8.8	190.7 $\pm$ 5.4	279 $\pm$ 11.6	0
DHB + lactate*	8.4 $\pm$ 0.2	2.3 $\pm$ 0.3	ND	20.9 $\pm$ 0.4
Catechol†	ND	3.03 $\pm$ 0.2	48.5 $\pm$ 8.2	0
<i>cis,cis</i> -Muconate	ND	0	196.5	0
β -Ketoadipate	ND	21.2 $\pm$ 1.7	ND	0
Lactate	0	0	0	0

* Lactate was added as DHB was not used as a growth substrate.

† Added in 0.5 mM increments.

spectroscopic analysis of the supernatant with a peak of absorption at 375 nm.

Carbon and energy fluxes for this culture prior to the period of catechol accumulation were estimated (Fig. 2b). Results with $q_{\text{benzoate}} = 5.2 \text{ mmol g}^{-1} \text{ h}^{-1}$ and $\mu = 0.3 \text{ h}^{-1}$ showed that maintenance represented 70% of the total energy (47.3 mmol ATP g⁻¹ h⁻¹ for a total of 67.5 mmol ATP produced g⁻¹ h⁻¹) and that a high flux (2.12 mmol g⁻¹ h⁻¹) through PDH was necessary.

Enzymic study of growth with 17–40 mM benzoate

The first four enzymes of the *ortho* pathway were assayed throughout the culture with 30 mM benzoate (Fig. 5). Results showed that B12O, DHBDH and C12O specific activities varied during the early hours of growth but remained constant after approximately 6 h of growth. It is interesting to note that DHBDH and C12O activities diminished during this transient period while B12O activity increased significantly. MCI specific activity showed a tenfold increase which took place throughout the entire duration of the culture. Similar observations were made for cultures with 17 and 40 mM benzoate.

Catechol accumulation. During the early stages of growth, it would appear that B12O limits benzoate degradation with a good correlation between the increase in B12O activity and specific rates of benzoate degradation. However, this situation changes radically once B12O has reached a maximum level. During this phase of the culture, the high B12O activity ($> 8 \text{ mmol g}^{-1} \text{ h}^{-1}$) together with the high affinity of this enzyme for its substrates, namely benzoate, oxygen and NADH (respective K_m values = 3.9, 4.3 and 10 μM as measured for the purified enzyme of *Pseudomonas arvilla* C-1; Yamagushi & Fujisawa, 1978, 1982) ensured a high rate of conversion of benzoate into DHB. Both DHBDH and C12O specific activities, which diminished during the first 6 h of the culture, stabilized thereafter. The specific activity of C12O was considerably lower than that of DHBDH, though similar to that reported by Johnson & Stanier (1971a).

The overflow of catechol would appear to indicate a pathway bottleneck localized at the C12O step. In view of the high affinity of C12O for catechol ($K_m = 1.5 \mu\text{M}$ for crude extracts of *A. entrophus* 335, this study; 0.3 μM for the purified enzyme of *A. entrophus* CH34, Sauret-Ignazi *et al.*, 1996), the enzyme was certainly substrate-saturated and hence the low level of expression was most probably the cause of this bottleneck. This hypothesis is further supported by the finding that C12O activity was not inhibited by the presence of benzoate, DHB or *cis,cis*-muconate. Thus the low activity of C12O limits the rate of catechol conversion to muconate and hence the overall flux through the *ortho* pathway under growth at benzoate concentrations in excess of 5 mM, but not exceeding approximately 40 mM. This enzyme was postulated to be induced by either benzoate or DHB (Johnson & Stanier, 1971b) though only low levels were induced when grown on lactate in the presence of DHB (Table 3), suggesting that benzoate is the principal inducer. Similarly, neither catechol nor *cis,cis*-muconate induced higher expression of C12O in *A. entrophus*.

MCI. MCI, the enzyme downstream of C12O in the *ortho* pathway, showed a characteristic increase in specific activity throughout the entire culture (Fig. 5). Its affinity for *cis,cis*-muconate is low ($K_m = 190 \mu\text{M}$ for crude extracts of *A. entrophus* 335, this study; $K_m = 55.8 \mu\text{M}$ for the purified enzyme of *Pseudomonas* B13, Meagher *et al.*, 1990; 100 μM for that of *P. putida*, Ornston, 1970). In addition, if the presence of organic acids (benzoate, DHB, but also lactate, acetate, succinate, fumarate, oxaloacetate, citrate, isocitrate, glyoxylate or formate) at concentrations of 1–10 mM had little or no effect on the conversion of muconate into muconolactone, catechol was found to be a strong competitive inhibitor ($K_i = 43 \mu\text{M}$) of MCI. Since no muconate was detected in the culture supernatant, even when high concentrations of catechol (up to 3.7 mM) were present, MCI specific activity was not limiting. It remains to be seen if the progressive increase in expression throughout the culture was necessary to maintain flux through the reaction in the presence of catechol.

DHB accumulation. Cultures for which significant accumulation of catechol was observed also showed DHB accumulation, though later in the fermentation. Since it has been shown that neither benzoate nor catechol influence activity of purified DHBDH (Reiner, 1972) *in vitro*, the simple enzyme equilibrium effect can be ignored. It was observed that DHB accumulation was often accompanied by trace amounts of hydroxybutyrate in the medium and the presence of polyhydroxybutyrate granules within the cell. These responses in *A. eutrophus* are generally interpreted as being symptomatic of a requirement for an additional electron sink (Steinbüchel & Schlegel, 1991). The obvious explanation of limiting oxygen availability for respiration needs to be examined, though pO_2 was at all times greater than 70% of air saturation. When measured, the NADH/NAD⁺ ratio was seen to increase gradually from an initial value of 0.08 during batch culture to 0.2 (Fig. 5). The impact on DHBDH activity was estimated *in vitro* with crude extracts of benzoate-grown cells, and the results showed a linear decrease to 50% activity at an NADH/NAD⁺ ratio of 0.5 as compared with NAD⁺ alone. Inhibition of various dehydrogenases of catabolic pathways by a high NADH/NAD⁺ ratio has been reported before (Snoep *et al.*, 1992; Girbal & Soucaille, 1994) but not to our knowledge for the enzymes involved in the catabolism of aromatic compounds. Affinities of B12O for NADH and of DHBDH for NAD⁺ have been determined to be 10 and 150 μ M, respectively (Reiner, 1972; Yamaguchi & Fujisawa, 1978). The ratio of these affinities (0.07) is close to the NADH/NAD⁺ ratio found at the start of the culture. However, the shift in the NADH/NAD⁺ ratio, possibly due to the inhibitory effect of catechol on respiratory activity (see above), would certainly contribute to the diminished *in vivo* activity of DHBDH. Supportive evidence for this stress condition may be seen in the increase in NADH oxidase activity which provides an alternative oxidation pathway under conditions in which energy excess conditions are encountered (Cocaign-Bousquet & Lindley, 1995) and thus avoids excess ATP production.

Induction of the catechol *meta*-cleavage pathway

The appearance of a yellow colouration of the medium during growth on high concentrations of benzoate has been described (Johnson & Stanier 1971a). However, no evidence for the induction of the *meta*-cleavage pathway has yet been reported despite the use of the appearance of a yellow colour (2-hydroxymuconic semialdehyde) as a test for the presence of this pathway. Several substrates and intermediates of the *ortho*-cleavage pathway were tested for their ability to induce the synthesis of C23O (Table 2). Catechol did not act as an inducer, contrary to what has been suggested by Johnson & Stanier (1971a). Indeed, it is logical that catechol cannot act both as an inducer (claimed by Johnson & Stanier, 1971a) and as a repressor (Hughes & Bayly, 1983) of C23O synthesis. Only DHB provoked the expression of C23O synthesis, though it is not clear whether DHB itself is the inducer since spontaneous decomposition of DHB to phenol or

salicylate may be occurring. However, no trace of phenol or salicylate could be detected in the supernatant, but this does not exclude the possibility that these compounds were present in the cell. C23O activity was found only towards the end of the culture period (a maximum specific activity of around 35 nmol mg⁻¹ min⁻¹ was measured, but was still increasing when the substrate was exhausted; Fig. 5), whereas an extremely low but constant phenol hydroxylase activity was detected in the cells throughout the culture period. It is interesting to note that Hughes & Bayly (1983) suggested a total repression of phenol hydroxylase by benzoate and catechol, and a 50% repression of the other enzymes of the *meta*-cleavage pathway by these compounds. Though our results suggest that the term total is perhaps exaggerated, such a control could explain the lack of induction of phenol hydroxylase by DHB. The accumulation of acetate, a product of the catechol *meta*-cleavage pathway, confirms that the other enzymes downstream of C23O in the *meta* pathway were also induced. The inability of *A. eutrophus* to convert acetate to acetyl-CoA is coherent with the repressed synthesis of acetyl-CoA synthetase observed during rapid growth on benzoate (Ampe & Lindley, 1995). The absence of acetyl-CoA synthetase activity was also confirmed here. However, the blockage of the pathway at this level provided a fortuitous manner by which flux distribution between the two pathways for the degradation of catechol could be estimated. Approximately 20% of the accumulated catechol was reconsumed through the *meta*-cleavage pathway. As both catechol dioxygenases have similar affinities for catechol and oxygen (see Fig. 7), the synthesis of C23O can be considered as a net increase in the amount of enzyme for the degradation of catechol.

Growth with 55 mM benzoate

When *A. eutrophus* was grown with 55 mM benzoate, the kinetic profile was different from that observed with 17–40 mM benzoate (Fig. 6). When growth still showed an accelerating pattern, values for the specific rate of benzoate consumption were much lower than those found in the experiments described previously, i.e. constant at approximately 3 mmol g⁻¹ h⁻¹ during the first 20 h of growth. The q_{benzoate} increased thereafter with accumulation of catechol and DHB when specific rates exceeded the value of 5.2 mmol g⁻¹ h⁻¹.

Inactivation of B12O

B12O activity was assayed by respirometry on whole cells throughout the various batch cultures as well as in the chemostat described above (Fig. 3b). Results showed that B12O activity was high (i.e. ≥ 9 mmol benzoate g⁻¹ cell dry wt h⁻¹, value always greater than the q_{benzoate} calculated for all the cultures) for benzoate concentrations up to 40 mM. Above this concentration, the specific activity fell sharply to 3 mmol benzoate g⁻¹ h⁻¹ or less, suggesting that the enzyme had been inactivated or its synthesis repressed. Cells grown in the presence of 55 mM benzoate regained high B12O activity when the concentration of the aromatic compound decreased. The

Fig. 6. Kinetics of growth and substrate consumption of *A. eutrophus* in batch culture on 55 mM benzoate. ■, benzoate; ◆, catechol; ○, DHB; ●, biomass; solid line, q_{benzoate} ; dotted line, μ .

other enzymes of the *ortho* pathway assayed did not appear to be affected (results not shown). Therefore, in the presence of high benzoate concentrations (over 40–45 mM), the conversion of benzoate into DHB by B12O becomes the limiting step for the degradation of the aromatic compound.

Conclusions

(1) Benzoate at low concentrations (< 5 mM) was a good substrate for the growth of *A. eutrophus* 335, but at higher concentrations up to 40 mM, growth was inhibited, but not the degradation of the aromatic compound. It may be possible to exploit this in waste water treatment or depollution strategies in which it is important to have high degradation yields with only minimal biomass production.

(2) The localization of metabolic bottlenecks in the *ortho* pathway were shown to change in response to the substrate concentration (results are summarized in Fig. 7). At low concentrations (around 5 mM), a maximum flux was observed through the *ortho*-cleavage pathway to-

Fig. 7. Summary of the regulation of the first steps of the *ortho*-cleavage pathway, and kinetic constants of the enzymes involved. Values are for: 1, *A. eutrophus* 335 and CH34 (this study); 2, *A. eutrophus* 335 (Reiner, 1972); 3, *P. arvilla* C-1 (Yamaguchi & Fujisawa, 1978); 4, *P. arvilla* C-1 (Yamaguchi & Fujisawa, 1982); 5, *P. aeruginosa* (Kataeva & Govlova, 1990).

gether with high efficiency fueling of an optimized central metabolism with regard to both anabolic precursor metabolites and the necessary co-enzyme requirements (i.e. low maintenance). For higher concentrations of up to approximately 40 mM, the bottleneck clearly appeared to be the *ortho*-cleavage of catechol into *cis,cis*-muconate catalysed by C12O, and perhaps as a consequence of this phenomenon though to a lesser extent, the conversion of DHB to catechol. Finally, at high concentrations (55 mM), the significantly diminished specific activity of B12O became limiting.

(3) The accumulation of DHB provoked the induction of the other major pathway for the degradation of catechol, the *meta*-cleavage pathway. We have presented here the first evidence for the role played by DHB in the induction of the *meta* pathway. Once this pathway had been induced, cells accumulated acetate, the product of the degradation of catechol via this pathway, as would be expected if the repression of acetyl-CoA synthetase activity by catechol (Ampe & Lindley, 1995) is taken into account.

(4) Pyruvate and the flux through pyruvate in *A. eutrophus* appear to play an important role in the efficiency of central metabolism. This is supported by the two following observations: (i) growth was very efficient when PDH was not necessary (Fig. 2a); and (ii) the induction of the *meta*-cleavage pathway on high concentrations of benzoate could also be interpreted as an extra source of pyruvate, one of the direct products of this pathway.

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