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Degradation of 2-bromo-, 2-chloro- and 2-fluorobenzoate by *Pseudomonas putida* CLB 250

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1. SUMMARY

Pseudomonas putida strain CLB 250 (DSM 5232) utilized 2-bromo-, 2-chloro- and 2-fluorobenzoate as sole source of carbon and energy. Degradation is suggested to be initiated by a dioxygenase liberating halide in the first catabolic step. After decarboxylation and rearomatization catechol is produced as a central metabolite which is degraded via the ortho-pathway. After inhibition of ring cleavage activities with 3-chlorocatechol, 2-chlorobenzoate was transformed to catechol in nearly stoichiometric amounts. Other *ortho*-substituted benzoates like anthranilate and 2-methoxybenzoate seem to be metabolized via the same route.

2. INTRODUCTION

Halogenated benzoic acids have served for a long time as model compounds for studying bacterial catabolism of halogenated aromatics [1–6].

In this context the *ortho*-substituted halobenzoates were of special interest as they proved to be more reluctant against biodegradation than the other isomers. Whereas a lot of a information on the metabolism of 2-fluorobenzoate is available [4], the other 2-halosubstituted benzoates were studied in much less detail. Utilization of 2-chlorobenzoate (2CB) by a *Pseudomonas cepacia* strain was claimed in the description of a patent [7]. However, by reexamination of the strain (kindly supplied by J.B. Robinson, Battelle Columbus Laboratories, Columbus, OH, U.S.A.) the catabolic potential described by the authors could not be reproduced. Another *Pseudomonas cepacia* strain [8] was described to utilize 2CB via catechol as an intermediate. The authors evidences against the involvement of other compounds in the degradation pathway (i.e. salicylate, chloromuconate), however, are ambiguous. Therefore studies were undertaken in order to broaden our knowledge on the mechanism of biodegradation of 2-halobenzoates. Some results obtained by an *Alcaligenes* species, strain CLB 280, have already been published [9]. This strain is currently further

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investigated. In the present communication a *Pseudomonas putida* strain is described and the results of the studies on biodegradation on 2-halobenzoates are discussed in comparison with the reports mentioned above.

3. MATERIALS AND METHODS

3.1. Organism

For enrichment purposes a mineral medium was used as described by Dorn et al. [5]. 2-Chlorobenzoate (2CB) without any additional vitamins was used as sole source of carbon and energy at later stages of isolation procedure. At the beginning 0.1% of yeast extract (Difco) was supplemented after sterile filtration.

3.2. Characterization

Most tests were performed as listed in the 'Manual of methods for general bacteriology' [10]. For species estimation the Api 20 NE test (Api-Merieux, Nürtingen, F.R.G.) was used and the results was evaluated by a computer with 20 NE-test specific software. The strain is deposited in the Deutsche Sammlung für Mikrobiologie, Braunschweig under the number DSM 5232.

3.3. Analytical methods, culture conditions, preparation of cell extracts, estimation of enzyme activities in crude extracts or intact cells

These have previously been described [11]. Spectra were recorded in situ during HPLC (see [12]; the eluent composition was modified to 35% methanol).

4. RESULTS AND DISCUSSION

4.1. Enrichment of strain CLB 250

Sewage sludge from the sewage treatment plant of Hoechst Ltd., Frankfurt was inoculated into a 2-chlorobenzoate containing mineral medium. After some weeks decrease of substrate concentration was monitored with HPLC. After transfer into fresh medium a well growing mixed culture was achieved. From this a pure strain CLB 250 was isolated by standard microbiological methods.

4.2. Characterization of strain CLB 250

Cells of strain CLB 250 were short, mobile rods. They were oxidase positive, Gram-negative and grew at 4°C but not at 37°C. Out of seventy non aromatic compounds tested, only glucose, fructose, mannose, glycerol, ribose, gluconate, esculine, caproate, malate and citrate were utilized as carbon sources. Metabolism of sugars was strictly aerobic, no growth was observed under fermentative conditions. From aromatic substrates tested, beside phenoxyacetate and benzoate also 2-fluoro-, 2-chloro- and 2-bromobenzoate, 2-methoxybenzoate and 2-aminobenzoate served as growth substrates. Salicylate, 2-methyl- and 2-iodobenzoate did not sustain growth. On Kings B agar plates a yellowish pigment was produced, the control strain *Pseudomonas putida arvilla* mt-2 showed identical behaviour. According to API 20 NE classification strain CLB 250 was tentatively identified as *Pseudomonas putida* with very high probability (99.9%).

Table 1

Utilization of 2-halobenzoates by *Pseudomonas putida* strain CLB 250

Growth substrate	Substrate concentration [mM] at		[s] [b]	[Hal^-] [c]	$\frac{[Hal]}{[s]} \times 100$	*td*[h]
	t_0 [a]	t_{10} [a]				
2-Fluorobenzoate	6.5	1.5	4	3 2 mM	80	4 5
2-Chlorobenzoate	5 1	0.1	5	4.9 mM	98	4
2-Bromobenzoate	4.2	0 7	3 5	3.5 mM	100	5

Cells were grown with the respective growth substrates.

[a] Substrate concentrations were determined with HPLC at time zero and after 10 h

[b] The amount of substrate consumed is given in mMol per litre

[c] Release of halides was measured by use of fluoride-, chloride- and bromide-sensitive electrodes respectively

Table 2

Enzyme activities in crude extracts of *Pseudomonas putida* strain CLB 250

Enzyme	Assay substrate	Specific activity (U/g protein) after growth with		
		succinate	2-chlorobenzoate	benzoate
1,2-dihydro-1,2-dihydroxybenzoate	DHB	< 10	1250	2110
Catechol-1,2-dioxygenase	Catechol	< 10	1970	2295
	3-Chlorocatechol	< 10	< 10	< 10
	4-Chlorocatechol	< 10	100	110
	3-Methylcatechol	< 10	120	150
	4-Methylcatechol	< 10	990	1200
Muconate cycloisomerase	Muconate	< 30	1260	400
	2-Chloro-*cis,cis*-muconate	< 30	< 30	< 30
4-Carboxymethylbut-3-en-4-olide hydrolase	4-Carboxymethyl-but-3-en-4-olide	< 30	600	885

Cells were grown with respective carbon sources at initial concentrations of 8–10 mM For description of enzymatic tests see MATERIALS AND METHODS. No activity could be detected for catechol-2,3-dioxygenase using catechol and 3-methylcatechol as substrates

4.3. Growth of CLB 250 with halobenzoates

Strain CLB 250 was able to mineralize all 2-substituted halobenzoates with the exception of 2-iodobenzoate (Table 1).

In the case of 2-chloro- and 2-bromobenzoate a rather good stoichiometry between substrate consumed and halide liberated was observed, whereas only 80% of the 2-fluorobenzoate was productively metabolized. This value was determined also with other organisms enriched on benzoates other than 2-fluorobenzoate. In these cases low regioselectivity of the initial dioxygenase allows substrate attack in 1,2- as well as in 1,6-position. The latter mechanism gives rise to the production of fluorosubstituted metabolites which are not completely metabolized.

4.4. Enzymes in crude extracts

All efforts to measure the activity of the proposed initial oxygenase enzyme in crude extracts with 2CB as a substrate were unsuccessful. (Cofactors used were NADH, NADPH, FADH and $FeSO_4$ alone and in combination). High activities, however, were found for 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase, catechol 1,2-dioxygenase, muconate cycloisomerase and 4-carboxymethylbut-3-en-4-olide hydrolase (see Table 2).

No activity could be detected for catechol-2,3-dioxygenase. Degradation of 2-chlorobenzoate (2CB) is therefore suggested to occur via unsubstituted catechol as a central intermediate (Fig. 1) which is metabolized by enzymes of an ortho cleavage pathway [14]. In addition, Table 3 shows other potential ring cleavage substrates not to be involved in the degradation pathway of 2CB. Furthermore, when ring cleavage of catechol was inhibited by adding 3-chlorocatechol [15], 2CB was temporarily transformed to catechol in nearly

Table 3

Relative oxygen uptake rates with 2-chlorobenzoate grown cells of *Pseudomonas putida* strain CLB 250

Substrate	Relative activity (%)
2-Chlorobenzoate	100
Catechol	260
4-Chlorocatechol	30
3-Chlorocatechol	< 3
2,3-Dihydroxybenzoate	< 3
3,4-Dihydroxybenzoate	8
2,5-Dihydroxybenzoate	< 3

Cells were grown with 2CB as sole source of carbon and energy (5 mM). For details of the oxygen electrode tests see (11). With the exception of the final substrate concentration was 1 mM for the benzoates and 0 5 mM for the catechols. The values are corrected for endogenous respiration.

stoichiometric amounts. Accordingly after co-metabolism of 3-chlorobenzoate (3-CB) excretion of chlorosubstituted catechols could be demonstrated to be also visually detectable by blue to violet coloration of the culture medium. These results confirm the proposals made for the *Pseudomonas cepacia* strain [8] that catechol is an intermediate of 2CB catabolism.

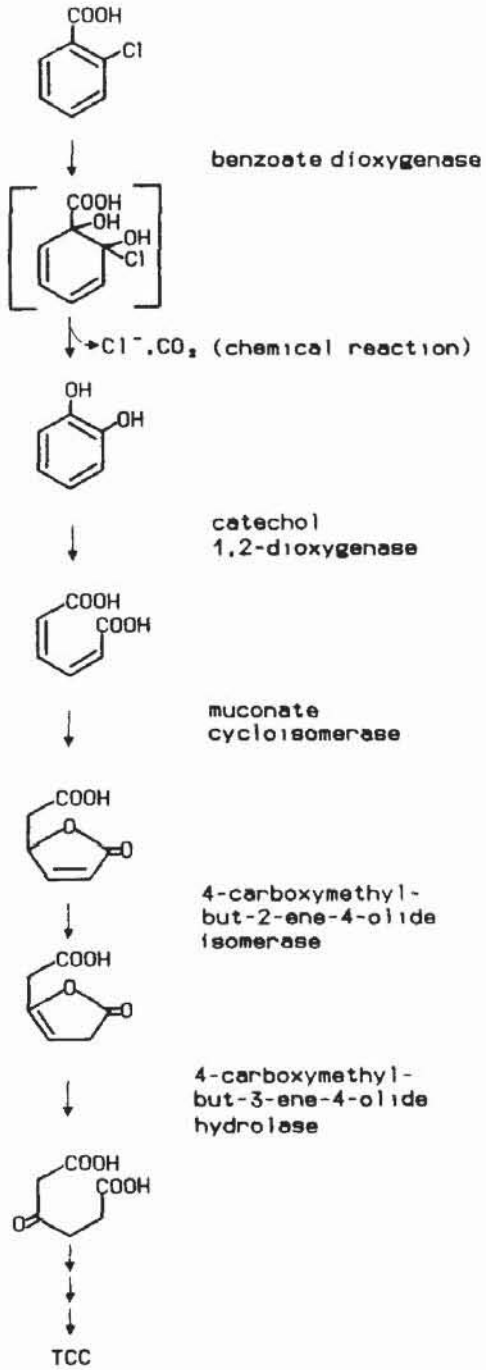

Fig 1 Degradation pathway of 2-chlorobenzoate in *Pseudomonas putida* CLB 250.

Table 4

Turnover of benzoate and substrate analogues by *Pseudomonas putida* strain CLB 250

Substrate	Relative rates of turnover after growth with	
	2-chloro-benzoate	benzoate
2-Chlorobenzoate	100	100
Benzoate	170	175
3-Chlorobenzoate	75	65
4-Chlorobenzoate	20	20
2-Fluorobenzoate	115	80
3-Fluorobenzoate	75	190
4-Fluorobenzoate	40	145
2-Bromobenzoate	35	< 5
2-Iodobenzoate	15	< 5
2-Methoxybenzoate	95	< 5
2-Ethoxybenzoate	80	< 5
2-Phenoxybenzoate	< 5	< 5
2-Methylbenzoate	40	20
3-Methylbenzoate	105	70
2-Hydroxybenzoate	20	35
2-Chloro-6-fluorobenzoate	5	< 5
2-Trifluoromethylbenzoate	< 5	n d
2-Sulphobenzoate	< 5	n.d

Cells of strain CLB 250 were grown with 2CB and benzoate respectively After suspension to an optical density 8 substrates were added to a final concentration of 3 mM. Decrease of substrate concentration was monitored with HPLC and the rates were calculated taking the value for 2CB as 100% n d, not determined

Catechol could be formed by action of a benzoate 1,2-dioxygenase specialized for turnover of 2-substituted benzoates. As in the case of the *Alcaligenes* species strain CLB 280 (9) a number of differently substituted benzoates were metabolized by 2CB grown cells of *Pseudomonas putida* CLB 250 (Table 4). Most interestingly, 2-methylbenzoate, a structural analogue of 2CB, seems to be metabolized to the same products as are formed by *Alcaligenes species* strain CLB 280 [9]. An extraordinarily acid-labile metabolite, (λ_{max} = 267 nm) suspected to be a methylsubstituted 1,2-dihydro-1,2-dihydroxybenzoate, after rearomatization yields *o*-cresol (data not shown). This would further substantiate the assumption of an 1,2-dioxygenase attack to initiate the metabolism of

2CB in *Pseudomonas putida* CLB 250. This metabolite which is not observed after cometabolism of 2-methylbenzoate by cells grown many generations with benzoate is currently investigated in detail. Salicylate (2-hydroxybenzoate) can be excluded as an intermediate in CLB 250 as (i) the strain shows only low activity with this substrate and (ii) does not grown with salicylate as a sole source of carbon and energy. From the data of Table 4 it can be deduced that two benzoate 1,2-dioxygenases can be differentiated by their substrate specificity. The enzyme present after growth on 2CB preferentially attacks 2-substituted benzoates. This problem is currently investigated in more detail as is the question whether 2-alkoxybenzoates ($C \geq 2$) serve as growth substrates in this organism. First results are indicating that alkoxy groups may be eliminated as corresponding alkanols after initial attack by the 2CB dioxygenating enzyme system. The same may be true for degradation of 2-aminobenzoate (anthranilate). This raises the interesting question whether 2-amino- or 2-methoxybenzoate generally are useful substrates for preenrichment of bacteria which gratuitously liberate halide from the aromatic ring by a dioxygenolytic mechanism. Studies to evaluate this possibility are currently undertaken.

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