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Enrichment of dibenzofuran utilizing bacteria with high co-metabolic potential towards dibenzodioxin and other anellated aromatics

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1. SUMMARY

Dibenzofuran degrading bacteria were enriched from various environmental sources. A mutualistic mixed culture of strain DPO 220 and strain DPO 230 was characterized. Strain DPO 220 alone showed limited growth with dibenzofuran as sole source of carbon and energy (td $\geq$ 4.5 h). A labile degradation product, $C_{12}H_{10}O_5$, and salicylate were isolated from the culture fluid. Salicylate was found to be a central intermediate of DBF-degradation.

Strain DPO 220 co-metabolized a wide range of anellated aromatics as well as heteroaromatics. High rates of co-oxidation of dibenzodioxin demonstrate analogue-enrichment to be a powerful technique for selecting enzymatic activities for otherwise non-degradable substrates.

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2. INTRODUCTION

As by-products of the synthesis of certain chlorinated aromatic compounds, polychlorinated dibenzofurans and dibenzodioxins (PCDF's and PCDD's) are the most spectacular accidentally dispersed environmental chemicals [1–6]. They now are widely distributed in the environment and cause great concern because of the extreme toxicity of some congeners, particularly 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD). Their potential to persist in the environment and to 'bioconcentrate' in animals and man is mostly due to the fact that these chemically highly stable molecules resist biodegradation. Degradation studies with prokaryotes are therefore rare [7–9].

In order to evaluate the existence in nature of marginal activities for the catabolism of these cyclic biaryl ethers we employed the proved strategy of using structural analogues for basic studies ([10], K.H. Engesser, W. Fietz and H.-J. Knackmuss. Abstr. Annu. Meet., VAAM, Konstanz, 1987, p. 30).

Dibenzofuran is the most simple cyclic biaryl ether and has been identified as a structural element in certain natural products [11–15]. Therefore in the present investigation it was used (i) as a structural analog for the enrichment of bacteria that oxidize dibenzodioxin or related compounds and (ii) as a model compound for elucidating mechanisms of cleavage of the biaryl ether bond. In addition, due to the extreme low solubility of DBF in aqueous systems (about 50 μM) its use as growth substrate required the development of new techniques of persuing its fate in the growing culture.

3. MATERIALS AND METHODS

DBF utilizing strains DPO 220, 1361, 233 and several mixed cultures used in this study were enriched using dibenzofuran as sole source of carbon and energy. Cells were grown at 30 °C in fluted Erlenmeyer flasks using mineral medium described previously [16]. With strains DPO 1361 and DPO 233 dibenzofuran was added after solubilization in an equal mixture of DMSO/Tween 80 (0.5% of culture volume). The final concentration was generally 10 mM if not otherwise noted. (The term 'concentration' was used throughout the paper also for substrate suspensions as if the compounds were totally dissolved.)

A complex medium of the following composition was used: KH_2PO_4 0.4 g, Na_2HPO_4 1.6 g, tryptone 4 g, yeast extract (Difco) 4 g, 4-aminobenzoic acid 2 g, glucose 1 g and water ad 1 l.

Growth was monitored photometrically by measuring the turbidity at 546 nm. In case of insoluble substrates, crystals were allowed to sediment for 1 min. Agar plates were prepared by adding the insoluble substrate in pulverized form to the agar before autoclaving. The sterile emulsion was allowed to cool while it was stirred vigorously to prevent formation of larger crystals. This suspension was poured into Petri dishes.

Pure cultures were kept on plates prepared by adding substrates solubilized in an equal mixture of DMSO and Tween 80 (0.5 ml in 1 l agar).

Strain DPO 220 was cultivated in continuous culture by use of a 1 l fermenter (Braun, Melsungen, F.R.G.) at 30 ° C. Two peristaltic pumps (BCC 2, BCC, Mannheim, F.R.G.) continuously fed the fermenter with mineral medium and withdrew the culture fluid. Dibenzofuran was added semi-continuously every 24 hours injecting 2 ml of a solution of DBF in acetone (50%), so that the initial concentration corresponded to 5 mM. The dilution rate was adjusted to $D = 0.05\ h^{-1}$ and the aeration was $1.5\ l/min^{-1}$.

Activities with whole cells were measured after resuspension of cells in phosphate buffer (50 mM, pH 7.4). If necessary, the culture fluid was passed through a plaited filter before centrifugation in order to remove substrate crystals. Oxygen uptake rates were measured using a Clark type oxygen electrode. Substrate was added as stock solution in phosphate buffer and DMSO for soluble and insoluble compounds respectively. The final concentration was adjusted to 5 mM.

3.1. Determination of concentration of insoluble substrates (See legend Fig. 2)

The supernatants were analyzed in a Merck HPLC system on a RP-8 licrosphere column (Bischof, Leonberg, F.R.G.; mobile phase water/methanol and water/acetonitrile acidified with H_3PO_4, respectively).

Preparative HPLC was carried out using a lichrosorb column (Bischof, Leonberg, F.R.G.; length 25 cm, diameter 2.5 cm, type 2025; in the mobile phase instead of H_3PO_4 TFA was used for pH adjustment). Methods for preparing extracts and determination of protein and enzymes have been published elsewhere [17]. For measurement of 2-hydroxymuconic semialdehyde dehydrogenase a previously published method [18] was modified using phosphate buffer (50 mM, pH 7.4). Gentisate dioxygenase was measured by following the increase of absorption at $\lambda = 331$ nm [19]. ^{1}H-NMR-Spectra were recorded at 300 MHz in deuterated acetonitrile with TMS as internal standard (model 32 k Formicy Transform, Bruker, Karlsruhe, F.R.G.). Mass spectra were recorded by a mass spectrometer model Bruker CXP with ASPECT-software (Bruker, Karlsruhe, F.R.G.). Chemicals were of the highest grade commercially available (Merck, Darmstadt, F.R.G.; EGA-Chemie, Steinheim, F.R.G. and Serva, Heidelberg,

F.R.G.). Biochemicals were from Boehringer, Mannheim, F.R.G.

4. RESULTS AND DISCUSSION

4.1. Isolation of bacteria

Dibenzofuran utilizing bacteria were enriched from various environmental samples. Firstly, activated sludge from a sewage plant of Weil der Stadt was used to isolate a yellow-coloured mixed culture consisting of strain DPO 220 and strain DPO 230 which could be subcultivated on DBF-mineral salts medium without any limitation. In contrast, strain 220 alone stopped growing on DBF as sole source of carbon and energy after one to two transfers. Inhibition of growth was always accompanied by the accumulation of dark polymers in the growth medium. Strain DPO 230 did not grow with DBF as a carbon source nor did it transform this compound to any measurable extent. Since a monoculture of DPO 220 could grow in continuous culture over a period of at least 25 generations the relationship in batch culture between this strain and strain DPO 230 cannot exclusively be interpreted as a mutualistic one. A possible role of strain DPO 230 in co-culture with DPO 220 could be the removal of a toxic metabolite produced by the latter organism.

Another DBF utilizing strain, strain DPO 1361, was isolated by a similar enrichment procedure from sediments and water of the river Rhine. In contrast to the syntrophic culture of strain 220 and 230, strain 1361 could grow in monoculture with DBF generating a similar yellow-brownish coloration of the medium. Dibenzofuran utilizing cultures could also be isolated from various industrial sewage plants, from the sewage treatment system of the University of Stuttgart at Büsnau as well as from a soil heavily contaminated with light fuel oil. With all these isolates an intense yellow coloration of the growing culture was observed.

4.2. Characterization of the isolates DPO 220 and 1361

Beside the characteristics given in Table 1, strain DPO 220 did not hydrolyze starch or gelatine, but utilized a wide range of sugars and acids as well as the aromatic substrates benzoate, salicylate, 2-indanone, fluorene and to some extent dibenzothiophene. Strain DPO 1361 cell wall preparations revealed directly linked mesodiaminopimelic acid, but neither mycolic acid nor N-glycolyl-esters. These results indicate that strain DPO 1361 belongs to the genus Brevibacterium.

Table 1

Characteristics of DPO 220 and DPO 1361

	DPO 220	DPO 1361
Mobility	–	–
Shape	r [1]	r
Rod-coccus cycle	–	+
Gram-reaction	+	+
KOH-test	–	–
Catalase	+	+
Oxidase	–	–
Acid from glucose	–	–
Lipase	+	n.d. [2]

[1] r = rod; [2] n.d. = not determined.

4.3. Growth of DPO 220 with dibenzofuran

Under certain conditions (see above) strain DPO 220 could be cultivated in batch culture with DBF as sole source of carbon and energy (Fig. 1). The maximal growth rate was calculated to be $\mu = 0.15\ h^{-1}$. Total consumption of the substrate is correlated with an increase in optical density of approximately 2.5 and the excretion of a small amount of a metabolite. The latter was extracted from the acidified cell free culture fluid and crystallized. It was found to have a molecular mass of m/z: 138. High resolution technique revealed an empirical formula of $C_7H_6O_3$. This together with ^{1}H-NMR, UV- and HPLC-data clearly identified the metabolite as salicylate. Salicylate was not excreted during growth of strain DPO 1361. HPLC-analysis of the culture supernatant, however, of both DBF-grown strains DPO 220 and 1361 revealed the excretion of three additional metabolites. The molecular mass of one of them was found to be m/z: 234.0527 corresponding to an empirical formula of $C_{12}H_{10}O_5$ with a maximal deviation of 0.0002 mass units. Comparison with the molecular formula of dibenzofuran ($C_{12}H_8O$) shows that 4 oxygen atoms have been inserted into the parent molecule together with 2 hydrogen

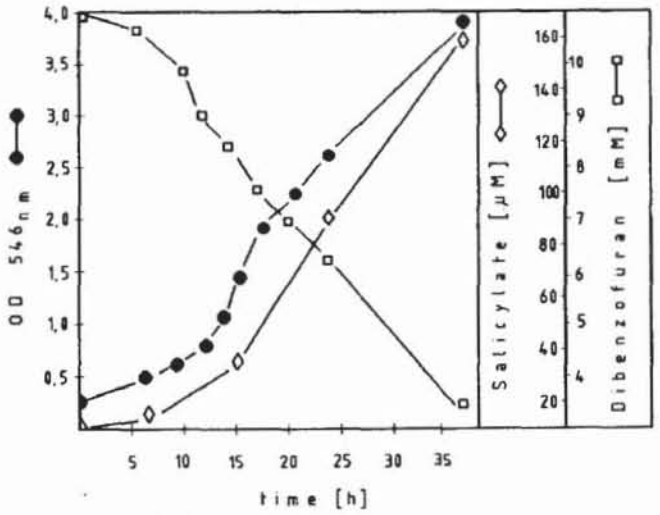

Fig. 1. Growth of strain DPO 220 with dibenzofuran as sole source of carbon and energy. Under the same conditions 10 Erlenmeyer flasks with 50 ml mineral medium containing dibenzofuran (0.5 mmoles = 10 mM) were inoculated with strain DPO 220 pre-grown with rich medium and incubated at 30 °C on a rotary shaker. With one flask the optical density was followed and the concentration of salicylate monitored by means of HPLC analysis. For every value of concentration determined one flask was processed as described in MATERIALS AND METHODS.

atoms. The analysis of ^{1}H-NMR-spectrum revealed two structural units: 4 protons could be assigned to an ortho-oxygen substituted benzoyl moiety (δ = 6.95 ppm,d; 7.81 ppm,d; 7.06 ppm,t; 7.53 ppm,t) as present in salicylic acid. The coupling pattern and the values of the coupling constants for the other protons indicated a 2-hydroxy-substituted propan unit flanked on each side by a carbonyl group. This indicates a structural reorganization of the furan-skeleton of DBF during metabolism. Due to the instability of the metabolite its ^{1}H-NMR spectrum was completely transformed into that of salicylic acid. The metabolite therefore contains a loosely attached, in ortho position oxygen-substituted benzoyl residue. From this it can be deduced that one of the benzenoid rings had been oxidized in such a way that the residual structure contains a labile ortho-oxo-benzoyl moiety.

The present data indicate that salicylate is a central metabolite of this pathway. Correspondingly a strong salicylate oxygenating activity can be measured with DBF grown cells of strain DPO 220 which exhibited the same level of induction as for DBF oxidation (relative oxygen uptake rate 93% compared with salicylate 100%). In order to explain the excretion of salicylate by these cells this metabolite must be generated from DBF at a high rate.

4.4. Salicylate as key intermediate

After growth with DBF cells of strain DPO 220 showed oxygen uptake not only with salicylate but also with catechol. Obviously salicylate is converted to catechol. Correspondingly, in extracts of DBF grown cells no activity of gentisate dioxygenase could be detected (see Table 2). Catechol as a substrate, however, was subject to meta-cleavage but not to ortho cleavage. The key enzymes of meta-cleavage pathway, both 2-hydroxy-

Table 2

Specific activities of the lower pathway enzymes in strain DPO 220 after growth with dibenzofuran and acetate

Enzyme/substrate		Specific activities (U/g) of cells grown with	
		DBF [1]	Acetate [2]
Gentisate dioxygenase			
Gentisate		< 5	< 5
Catechol 1,2-dioxygenase			
Catechol		< 5	< 5
Catechol 2,3-dioxygenase			
Catechol		170	7
3-Methylcatechol		100	25
4-Methylcatechol		70	12
2-Hydroxymuconic semialdehyde dehydrogenase			
2-Hydroxymuconic	−NAD	< 2	< 2
semialdehyde	+NAD	57	< 2
5-Methyl-2-hydroxy-muconic	−NAD	2	< 2
semialdehyde	+NAD	31	< 2
2-Hydroxy-6-oxohepta-2,4-dienoate hydrolase			
2-Hydroxy-6-oxohepta-	−NAD	28	< 4
2,4-dienoate	+NAD	31	< 4

For preparing extracts, determination of protein and estimation of enzyme activities see MATERIALS AND METHODS.

[1] Cells were grown with dibenzofuran as sole carbon and energy source which was suspended as crystals in the growth medium (15 mM suspended).

[2] Cells were grown for at least 20 generations with acetate (5 mM).

muconic semialdehyde dehydrogenase and 2-hydroxy-6-oxo-hepta-2,4-dienoate hydrolase were induced in DBF grown cells of strain DPO 220.

4.5. Elements of a DBF degradation pathway

Following the general principle of degradation of anellated aromatics [20–22] the formation of the $C_{12}H_{10}O_5$-product should be mediated by two dioxygenases. Cleavage of the hydroxylated ring then would yield a two ring structure in which the ether oxygen bond has become labile.

2-Coumaranone (see Fig. 2) was used as a probe for detecting enzymes which hydrolyze 2-hydroxybenzofuran structures. After growth on DBF strain DPO 220 induced a significantly higher activity of a 2-coumaranone hydrolase when compared with cells grown on complex medium (rel. act. 250% compared with non-induced cells 100%). Specific induction of this enzyme activity supports the hypothesis that ether bonds may be susceptible to hydrolysis after being transformed to ester bonds [23,24].

4.6. Cometabolic potential of dibenzofuran degrading bacteria

After growth with DBF cells of strain DPO 220 turned over a broad range of anellated aromatics and heteroaromatics (Fig. 2). The compounds not metabolized were: 9-carboxyfluorene, 1-indanone, 3-formyl-2-coumaranone and homophthalate. Phthalate (27%), salicylate (100%) and *o*-hydroxyphenylacetate (32%) were metabolized (percentage given in brackets). In no case was substrate degradation complete as was shown by the accumulation of metabolites (followed by HPLC) and/or by intensive coloration of the medium.

These experiments demonstrate the usefulness of analogue enrichment technique for amplifying marginally existing enzyme activities that turn over poorly biodegradable compounds. In case of highly toxic and recalcitrant chemicals like 2,3,7,8-TCDD (2,3,7,8-Tetrachlorodibenzodioxin) the enhance-

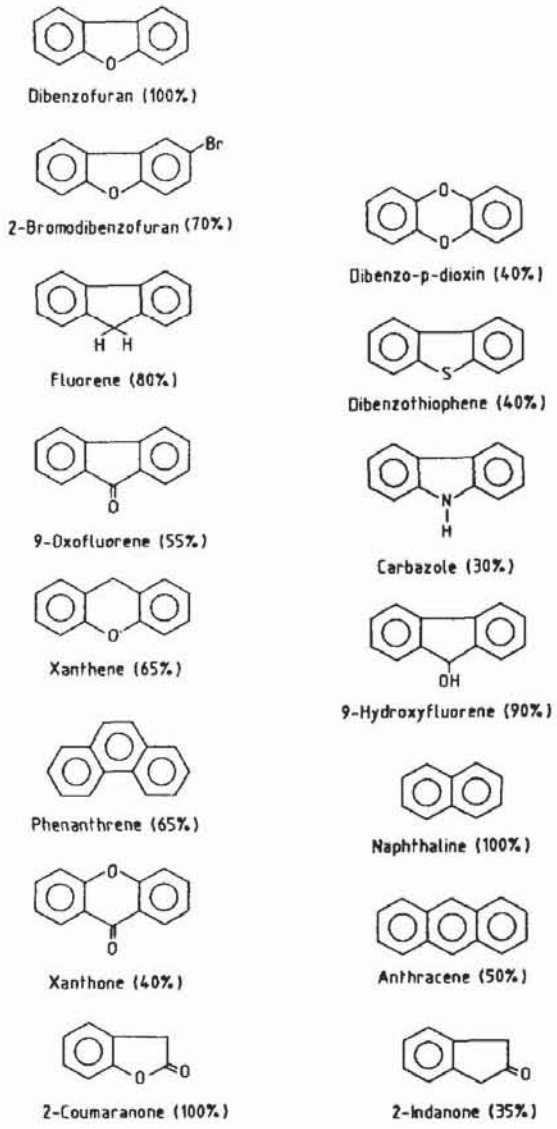

Fig. 2. Co-metabolism of aromatics by dibenzofuran grown cells of strain DOP 220. For co-metabolism experiments cells of DPO 220 were grown with DBF as the sole carbon source. Cells were resuspended in Erlenmeyer flasks containing phosphate buffer (pH 7.4, 50 mM; OD546 = 5) and incubated in a rotary shaker (30 °C). Substrates (5 μmoles = 1 mM) were added as stock solutions in DMSO (100 mm). For each substrate two flasks were used for determination of concentration after an incubation period of 1 and 60 minutes. The percentage of substrate degraded in the flask incubated for 60 min compared to the reference flask is given in brackets. Metabolism was stopped by adding dioxan (20 ml) and substrate concentration was determined after centrifuging down the precipitated protein. Control experiments with heat inactivated cells (10 min 80 °C) excluded adsorption affects being responsible for decrease of substrate concentration. Coincidentally, with dibenzofuran, dibenzodioxin and fluorene uptake of oxygen was determined. With the exception of 2-coumaranone and 2-indanone yellow to orange coloration of the medium was observed in all substrates.

ment of initial co-metabolic transformations may be the crucial step for starting the biological process of reclamation of dump sites or polluted soils.

Since preliminary experiments on the metabolism of dichlorinated dibenzodioxins by such dibenzofuran degrading cultures (especially DPO 233) were successful, further efforts will concentrate on the initial attack of the aromatic ring, its dechlorination as well as the general mechanisms of cleavage of the aromatic ether bond.

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