Appl Microbiol Biotechnol (2002) 59:62–67
DOI 10.1007/s00253-002-0979-7

ORIGINAL PAPER

M. B. Stope · D. Becher · E. Hammer · F. Schauer

Cometabolic ring fission of dibenzofuran by Gram-negative and Gram-positive biphenyl-utilizing bacteria

Received: 12 October 2001 / Revised: 12 February 2002 / Accepted: 15 February 2002 / Published online: 6 April 2002

Abstract Thirty-five strains of soil bacteria were grown with biphenyl (BP) and tested for their capacity to cooxidize dibenzofuran (DBF). During metabolism of DBF, the culture medium of 17 strains changed from colorless to orange, indicating a *meta*-cleavage pathway of DBF degradation. The ring cleavage product of these isolates was shown to be 2-hydroxy-4-(3′-oxo-3′*H*-benzofuran-2′-yliden)but-2-enoic acid (HOBB). The strain SBUG 271, studied in detail and identified as *Rhodococcus erythropolis*, degraded DBF via 1,2-dihydroxydibenzofuran. The ensuing *meta*-cleavage yielded HOBB and salicylic acid. In addition, the four monohydroxylated monomers of DBF and two metabolites, which were not further characterized, were detected. Thus, our results demonstrate that the metabolic mechanism involves lateral dioxygenation of DBF followed by *meta*-cleavage and occurs in Gram-negative as well as in Gram-positive BP-degrading bacteria.

Introduction

Dibenzofuran (DBF) and the halogenated congeneres of DBF are reported to be environmental pollutants with potential risks for human health (Goerlitz et al. 1985; Ahlborg et al. 1992; van Birgelen et al. 1996). In recent years, several strains of bacteria have been isolated and characterized which are able to use DBF as a growth substrate. Most of these strains are members of the proteobacteria, namely *Pseudomonas* sp. NCIB 9816–4 (Resnick and Gibson 1996), *Pseudomonas fluorescens* TTC1 (Bianchi et al. 1997), *Sphingomonas* sp. HH69 (Fortnagel et al. 1990; Harms et al. 1995) and strain RW1 (Bünz et al. 1993; Wilkes et al. 1996). Additionally, Gram-positive bacteria like *Brevibacterium* sp. DPO1361 (Engesser et al. 1989), *Terrabacter* sp. DBF63 (Monna et al. 1993; Kasuga et al. 1997) and strain DPO360 (Schmid et al. 1997) seem to be able to oxidize DBF. The DBF degradation pathway of these bacteria starts with hydroxylation at carbon atoms 4 and 4a (angular dioxygenation), resulting in the cleavage of the ether bridge, and is followed by formation of 2,2′,3-trihydroxybiphenyl (Strubel et al. 1991). Subsequently, a second dioxygenase catalyzes the ring cleavage of this intermediate to 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoic acid, a *meta*-cleavage product, which is transformed to 2-hydroxypenta-2,4-dienoic acid and salicylic acid (Fortnagel et al. 1990; Bertini et al. 1995).

Ring cleavage of DBF after hydroxylation at carbon atoms 1 and 2 (lateral dioxygenation) by biphenyl (BP)-utilizing bacteria has been postulated by several authors, because of the yellow-orange coloration during incubation with the substrate (Cerniglia et al. 1979). This assumption has been proven for *Ralstonia* sp. SBUG 290, which degraded DBF after cultivation with BP. Ring fission of 1,2-dihydroxydibenzofuran (1,2-DiOH-DBF) resulted in production of 2-hydroxy-4-(3′-oxo-3′*H*-benzofuran-2′-yliden)but-2-enoic acid (HOBB), which was degraded via salicylic acid (Becher et al. 2000).

The aim of our study was to investigate whether the lateral dioxygenation of DBF with subsequent *meta*-cleavage of the dihydroxylated intermediate can be carried out by other Gram-negative or Gram-positive bacteria growing with BP. The DBF metabolism of the Gram-positive isolate *Rhodococcus erythropolis* SBUG 271 has been characterized in more detail.

M.B. Stope (✉)
Institute of Molecular Biology, Friedrich-Loeffler-Institutes,
Federal Research Centre for Virus Diseases of Animals,
Boddenblick 5a, 17498 Insel Riems, Germany
e-mail: stope@rie.bfav.de
Tel.: +49-38351-7267, Fax: +49-38351-7151

D. Becher · E. Hammer · F. Schauer
Institute of Microbiology and Molecular Biology,
Ernst-Moritz-Arndt-University of Greifswald,
Friedrich-Ludwig-Jahnstrasse 15, 17487 Greifswald, Germany

Materials and methods

Organisms and growth conditions

The bacteria were isolated from sewage sludge and compost soil samples by enrichment cultivation performed in 500-ml flasks

containing 100 ml mineral salts medium (5 g $NH_4H_2PO_4$, 2.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.46 g K_2SO_4, 0.07 g $CaCl_2$, 2 mg $FeCl_3 \cdot 6H_2O$, 0.5 mg H_3BO_3, 0.1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg KI, 0.4 mg $MnSO_4 \cdot 5H_2O$, 0.4 mg $ZnSO_4 \cdot 7H_2O$, 0.2 mg Na_2MoO_4, 0.1 mg $CoCl_3$ per liter of deionized water) according to Hundt et al. (1998) and BP (2 mg ml^{-1}), 4-chlorobiphenyl (0.2 mg ml^{-1}), or 2-hydroxybiphenyl (0.2 mg ml^{-1}) as the only source of carbon as described by Becher (1997). Primary differentiation of isolated strains was by Gram-staining, microscopy of cells, and colony morphology after cultivation on six different solid media. Several subsequent biochemical tests were performed according to *Bergey's manual of determinative bacteriology* (Holt et al. 1994) to verify that all tested isolates were members of different species.

Stock cultures of the strains were maintained on nutrient broth agar (Sifin, Berlin, Germany) and all strains used are deposited as frozen stocks at –80°C in the strain collection of the Department of Biology of the University of Greifswald (SBUG). The strain SBUG 271 is deposited at the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (no. DSM 44606).

Cultivation with BP (1 mg ml^{-1}) was carried out in 500-ml flasks containing 100 ml mineral salts medium for 48–96 h at 30°C and 180 rpm (Becher et al. 2000).

For DBF incubation experiments, BP-grown cells were harvested by centrifugation (30 min, 15,000 *g*) and washed twice with sodium phosphate buffer (67 mM, pH 7.0). The pellet was resuspended in mineral salts medium at an optical density of 5.0 (600 nm) and inoculated into DBF (0.25 mg ml^{-1})-containing medium. Supplemented flasks without cells were used as controls.

Identification of metabolites

Aqueous supernatants of bacterial cultures were analyzed by high-performance liquid chromatography (HPLC) using a Hewlett-Packard 1050M apparatus (Bad Homburg, Germany) with a diode array detector operating at 220 nm and a LiChroCart 125–4 RP-18 endcapped (5 µm) column (Merck, Darmstadt, Germany). HPLC was performed according to the method of Hammer et al. (1998). Methanol and phosphoric acid (0.1%) were used as eluents at an initial ratio of 30:70 (pH 3.0) reaching 100% methanol after 14 min at a flow rate of 1 ml min^{-1}.

Gas chromatography-mass spectrometry (GC-MS) analyses were carried out with samples obtained by extraction of the aqueous supernatants with ethyl acetate at pH 7.0 and pH 2.0, respectively. Evaporated extracts were dissolved in methanol and derivatized by methylation with diazomethane (De Boer and Backer 1956). The methylated samples were analyzed using a GC-MS coupling system (GC 8000 and a mass spectrometer MD 800, Fisons Instruments, Mainz, Germany). Separation was obtained on a 30 m DB5-ms (0.25 mm i.d.) column (J&W Scientific, Folsom, Calif.), with a temperature program from 80°C to 300°C at 10 K min^{-1}.

Detection of orange ring fission products

For detection of the colored ring fission products on solid media, isolates were cultivated on nutrient agar for 24 h at 30°C. A colorless aqueous solution of 5 mM 2,3-dihydroxybiphenyl (2,3-DiOH-BP) or 5 mM 1,2-DiOH-DBF was sprayed over the bacterial colonies and incubated for 10 min. A change in color of the colonies to bright yellow or orange indicated the ring fission of the dihydroxylated substrates (Lunt and Evans 1970; Cerniglia et al. 1979; Foght and Westlake 1988; Becher et al. 2000).

Results

Isolation and characterization of bacterial strains

Thirty-five bacterial strains were isolated from different sewage sludge and compost soil samples by enrichment cultivation with BP and different BP derivatives as carbon source. All isolated strains were able to utilize BP as growth substrate and 17 strains showed the ability of cometabolic DBF ring fission after BP cultivation in liquid medium (Table 1). These 17 isolates were used for further characterization of DBF degradation as described below.

All 17 isolates were characterized for a number of taxonomically relevant features including Gram-reaction, cell and colony morphology, and physiology (e.g., oxidase and catalase reaction; utilization of glucose and citrate, nitrate reduction to nitrite and denitrification) to check if the individual strains belong to different bacterial species. The Gram-positive strain SBUG 271 was identified in cooperation with R. M. Kroppenstedt (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany) as *R. erythropolis* by analysis of peptidoglycan-type, mycolic acid structure, and fatty acid pattern.

Table 1 Gram-reaction, isolation substrates and origin of isolated bacteria able to cooxidize dibenzofuran (DBF)

Strain	Gram-reaction	Carbon source of isolation	Origin of sample
SBUG 265	–	2-OH-BP	Sewage sludge
SBUG 266	–	biphenyl (BP)	Sewage sludge
SBUG 268	–	4-Cl-BP	Sewage sludge
SBUG 278	–	BP	Sewage sludge
SBUG 292	–	BP	Sewage sludge
SBUG 301	–	4-Cl-BP	Sewage sludge
SBUG 311	–	4-Cl-BP	Sewage sludge
SBUG 263	+	BP	Sewage sludge
SBUG 264	+	BP	Sewage sludge
SBUG 267[a]	+	BP	Compost soil
Rhodococcus erythropolis SBUG 271	+	BP	Sewage sludge
SBUG 273	+	4-Cl-BP	Compost soil
SBUG 280	+	BP	Sewage sludge
SBUG 282	+	4-Cl-BP	Compost soil
SBUG 284	+	4-Cl-BP	Compost soil
SBUG 287[a]	+	BP	Sewage sludge
SBUG 288	+	4-Cl-BP	Compost soil

[a] Show high physiological similarities

Table 2 Ring cleavage activities of isolated bacteria after precultivation on bacto nutrient agar or in liquid BP medium with 2,3-DiOH-BP, 1,2-DiOH-DBF, or DBF as substrate

Strain	Precultivation	Nutrient agar		Liquid BP medium	
	Incubation substrate	DiOH-BP	DiOH-DBF	DBF	
	Ring cleavage indicated by	Yellow colonies	Orange colonies	Orange medium	HPLC analysis
SBUG 265		–	–	+	+
SBUG 266		+	+	+	+
SBUG 268		+	–	+	+
SBUG 278		+	+	+	n.d.[a]
SBUG 292		+	+	+	n.d.
SBUG 301		+	+	+	n.d.
SBUG 311		+	+	+	n.d.
SBUG 263		+	+[b]	+	+
SBUG 264		+	–	+	+
SBUG 267		+	+	+	n.d.
Rhodococcus erythropolis SBUG 271		+	+	+	+
SBUG 273		+	+	+	n.d.
SBUG 280		+	+	+	n.d.
SBUG 282		+	+	+	+
SBUG 284		+	–	+	+
SBUG 287		+	–	+	+
SBUG 288		+	+	+	n.d.

Growth experiments with BP and DBF

All isolates examined grew with BP as substrate and reached stationary phase after 72–120 h, at an optical density at 600 nm of 1.0–2.0. Besides a small amount of benzoic acid, which was detected over a short period of time only, no other metabolites were found in the culture supernatants of BP culture. There was no visible coloration of culture media, except in the case of BP-cultivation of strain SBUG 278 and strain SBUG 292, which resulted in a short-lived yellow-orange coloration of the medium. None of these strains were able to use DBF as sole source of carbon and energy.

Examination of DBF-transformation

In order to investigate the possibility of cometabolic DBF degradation, all 17 strains were precultivated in liquid medium with BP (1.0 mg ml^{-1}) and, after centrifugation, the resuspended cells were transferred to liquid medium with DBF (0.25 mg ml^{-1}). All isolates showed the orange ring cleavage product during incubation with medium containing DBF (Table 2, liquid BP medium), indicating a *meta*-cleavage pathway of cometabolic DBF transformation. Since the rapidly formed color of the medium disappeared after 20–60 h, we believe this product to be an intermediate rather than a dead-end product. Controls without cells showed neither coloration of medium nor formation of transformation products.

For characterization of the orange ring fission product formed from DBF after BP preculture, we analyzed 100-µl samples of the aqueous supernatants of different strains with HPLC. The major *meta*-cleavage product of DBF was identified as HOBB, already found in a *Ralstonia* strain by Becher et al. (2000), by comparison of the HPLC retention time (RT) and the UV absorption spectrum with that of an authentic standard. From 17 strains examined, 9 were positive for HOBB formation (Table 2, liquid BP medium), indicating lateral dioxygenation followed by *meta*-cleavage of DBF. Although coloration of the medium was also observed in the case of the other eight strains, no responsible substances could be detected by HPLC-UV analysis.

The kinetics of formation of HOBB and some other metabolites were studied in *R. erythropolis* SBUG 271 because this strain accumulated metabolites in greater amounts and showed a faster formation of colored ring fission product than the other strains examined. HPLC analysis of the aqueous supernatant of *R. erythropolis* SBUG 271 incubated with DBF after BP precultivation revealed three major metabolites in addition to several products present at low concentration. During incubation, HOBB (RT =8.9 min) was rapidly formed, accompanied by an intensive orange coloration of the culture medium, followed by a rapid degradation of this compound (Fig. 1).

The formation of an orange product in the culture medium during DBF transformation of *R. erythropolis* strain SBUG 271 indicated its production by a *meta*-cleavage pathway. The absorption maxima shifted from λ_{max} =462 nm at pH 7.0 to λ_{max} =400 nm at pH 3.0 (Fig. 2), as a result of keto-enol tautomerism. These characteristics are identical to those reported for HOBB by Becher et al. (2000). DBF was rapidly metabolized to HOBB, reaching a calculated transformation rate of 16% after an incubation time of 7 h.

The remaining two metabolites, M1 (RT =4.4 min) and M2 (RT =6.0 min), accumulated more slowly over the whole incubation time (Fig. 1). The UV spectra

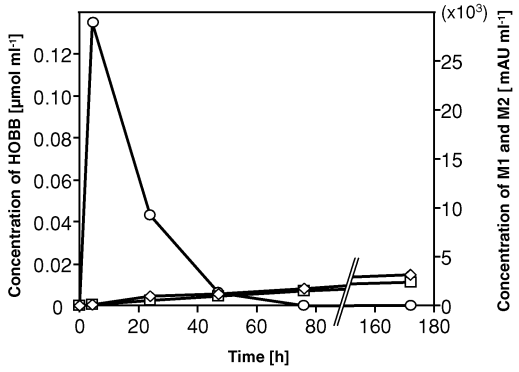

Fig. 1 Formation of the major metabolites 2-hydroxy-4-(3′-oxo-3′*H*-benzofuran-2′-yliden)but-2-enoic acid (*HOBB*), *M1*, and *M2* during dibenzofuran (DBF) incubation of biphenyl (BP)-grown cells of *Rhodococcus erythropolis* SBUG 271. High-performance liquid chromatography (HPLC) analysis of aqueous supernatants after the indicated incubation times. Retention times (RT) are shown in brackets. The initial concentration of DBF was 1.49 µmol ml^{-1}. *Circles* HOBB (RT =8.9 min), *diamonds* M1 (RT =4.4 min), *squares* M2 (RT =6.0 min). The amount of M1 and M2 was calculated from their absorbance at 220 nm. *AU* Absorbance units

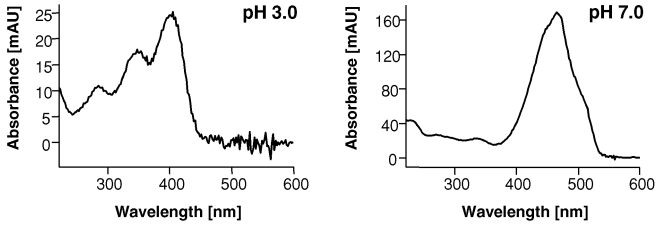

Fig. 2 UV-Spectra of the DBF metabolite HOBB of *R. erythropolis* SBUG 271 at pH 3.0 (left) and at pH 7.0 (right). *AU* Absorbance units

showed absorption maxima of λ_{max} =258 nm for M1 and λ_{max} =256 nm for M2. However, the low concentration and the low stability of these compounds during enrichment and purification procedures prevented further characterization.

After GC-MS analysis of the extracts, six additional products were found. These products were identified by specific RT and mass spectra as being a DiOH-DBF which was not further characterized, salicylic acid and the four isomers of monohydroxylated DBF as already described for *Ralstonia* sp. SBUG 290 (Becher et al. 2000).

To distinguish between constitutive or induced expression of the ring fission enzymes, all 17 isolated strains were cultivated on nutrient agar followed by a short incubation (30 s to maximally 10 min) of the grown colonies with the dihydroxylated precursors 2,3-DiOH-BP and 1,2-DiOH-DBF. Color formation by bacterial colonies indicated a *meta*-ring fission of the substrates; 16 strains rapidly transformed the dihydroxylated BP and 12 (all except strains SBUG 264, 268, 284, and 287) were additionally able to transform the 1,2-DiOH-DBF to orange products (Table 2, nutrient agar). These results may hint at constitutive synthesis of ring fission dioxygenases in most of the isolates. Using this technique, no ring fission products were detectable during short incubations of the strain SBUG 265.

Discussion

Of 35 bacterial isolates growing with BP as the only source of carbon and energy, 17 were able to degrade DBF cometabolically. The ring cleavage of DBF was accompanied by an orange coloration of the culture medium, caused by the DBF *meta*-cleavage product. The formation of colored degradation products during cometabolic degradation of DBF has been described previously (Cerniglia et al. 1979; Grifoll et al. 1995), but no chemical characterization of the structure of the ring cleavage product or other metabolites was provided. This was probably a result of the low absorbance of these products and the resulting low response factor at 220 nm or 254 nm, the wavelengths commonly used for UV detection of aromatic compounds. Furthermore, low concentrations of the intermediates or instability of these compounds during acidic elution conditions might have prevented further characterization. Selifonov et al. (1991) described the formation of 2-oxo-4-(3′-methoxybenzofuran-2′-yl)but-3-enoic acid in a *Pseudomonas* strain, which represents the methyl derivative of formula IX shown in Fig. 3, during cometabolic degradation of DBF. The ring fission product of this *Pseudomonas* strain accumulated as a dead-end product due to the lack of cleavage of pyruvate from it by the hydrolase. Because of these data, it was assumed that only bacteria using DBF as sole source of carbon and energy via an angular oxidation mechanism are able to degrade DBF completely. Lateral oxidation leads to dead-end products, namely the dihydroxylated DBF derivative or, extremely rarely, a ring cleavage product (Wittich 1998). Our study shows, however, that the mechanism of lateral dioxygenation followed by *meta*-cleavage of DBF seems to be widely distributed among biphenyl-degrading bacteria.

Seven Gram-negative and ten Gram-positive strains incubated after BP precultivation with DBF showed the characteristic coloration of the medium, indicating DBF ring fission via *meta*-cleavage.

The ring cleavage product HOBB was detected via HPLC in the aqueous supernatants of the strains SBUG 263, 264, 265, 266, 268, *R. erythropolis* SBUG 271, 282, 284, and 287. In the case of the remaining eight strains, despite coloration of the medium indicating the formation of a *meta*-cleavage product, this intermediate could not be detected using HPLC analyses. This may be due to the same analytical difficulties described above (low absorbance, low concentration).

To collect some data on the regulation of the BP and DBF ring cleavage enzymes, we incubated nutrient agar-grown bacterial colonies with 2,3-DiOH-BP and 1,2-DiOH-DBF without BP precultivation. Twelve strains formed colored ring fission products in the pres-

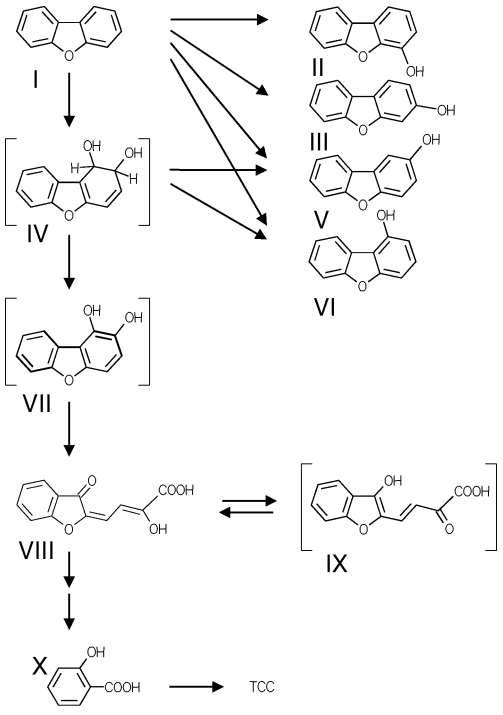

Fig. 3 Proposed main pathway of DBF degradation in *R. erythropolis* SBUG 271. Compounds in *square brackets* were not detected: *I* DBF, *II* 4-hydroxydibenzofuran, *III* 3-hydroxydibenzofuran, *IV* 1,2-dihydro-1,2-dihydroxydibenzofuran, *V* 2-hydroxydibenzofuran, *VI* 1-hydroxydibenzofuran, *VII* 1,2-DiOH-DBF, *VIII* HOBB, *IX* 2-oxo-4-(3′-hydroxybenzofuran-2′-yl)-but-3-enoic acid, *X* salicylic acid, *TCC* tricarboxylic acid cycle

ence of the dihydroxylated precursors, and no color formation at all was observed only after incubation of strain SBUG 265. These results suggest constitutive expression of the BP and DBF ring cleaving enzymes in the former strains and inducible synthesis of these enzymes in the latter strain. It is noteworthy that strains SBUG 264, 268, 284, and 287 grown on nutrient agar produced orange ring fission products in the presence of 2,3-DiOH-BP, but that no ring fission of 1,2-DiOH-DBF was detectable, suggesting the possible existence of at least two dioxygenases with a varying substrate specificity in this strain.

A more detailed characterization of DBF metabolism by BP precultivated cells of *R. erythropolis* SBUG 271 revealed that the cometabolic pathway of DBF proceeds via an initial dihydroxylation of the substrate at the carbon atoms in positions 1 and 2 and a subsequent *meta*-cleavage of the molecule. HPLC and GC-MS analysis of culture supernatants and ethyl acetate extracts revealed one dihydroxylated DBF derivative, HOBB, salicylic acid and the four possible monohydroxylated DBF derivatives. The formation of 1-, 2-, 3-, and 4-hydroxydibenzofuran could be explained either by the spontaneous conversion of the corresponding unstable dihydrodiols (Cerniglia et al. 1979; Resnick and Gibson 1996), or by an unspecific or specific monooxygenase activity, which has so far not been described for bacterial degradation of biaryl compounds. Both of these possible reactions would result in dead-end products. The presence of HOBB could only be explained by *meta*-cleavage of the 1,2-dihydroxylated precursor between the carbon atoms in positions 1 and 9b. This hypothesis is supported by the degradation of 1,2-DiOH-DBF by *R. erythropolis* SBUG 271, showing the typical orange coloring of culture medium as a result of *meta*-cleavage. The detection of other, partly unidentified compounds, indicated the existence of additional DBF pathways or intermediates. Because *R. erythropolis* SBUG 271 was capable of growing with salicylic acid as a carbon source, partial mineralization of DBF is at least conceivable. The proposed main pathway (Fig. 3) shows that, in addition to *Ralstonia* sp. SBUG 290 (Becher et al. 2000), the Gram-positive bacterium *R. erythropolis* SBUG 271 is also able to degrade DBF using the same, or a very similar, pathway as shown for *Ralstonia*. The proposed pathway is closely related to the bacterial degradation of dibenzothiophene, a compound very similar to DBF. Kodoma et al. (1973) described the cometabolic degradation of dibenzothiophene, starting with 1,2-dihydroxylation and subsequent *meta*-cleavage. The ring fission product was transformed to dead-end products, the corresponding hemiacetal and 3-hydroxy-2-formyl-benzothiophene.

The results of preliminary experiments on the regulation of the ring fission enzymes lead to the conclusion that there is a connection between the BP- and the DBF-degrading sequences. It is conceivable that the cleavage of both dihydroxylated precursors is carried out by the same ring cleavage enzyme or that the BP and DBF ring cleavage enzymes are coregulated. Preliminary enzymological investigations revealed that *R. erythropolis* SBUG 271 and *Ralstonia* sp. SBUG 290 expressed a constitutive BP ring cleavage dioxygenase with an additional DBF ring cleavage activity. After separation of cellular proteins, both BP and DBF ring cleavage activities were detectable in the same protein fraction (unpublished data). Thus, there is evidence that some of the BP degradation enzymes may be involved in special steps of the DBF degradation pathway.

Acknowledgements We would like to thank Michael Specht (Hamburg, Germany) for synthesis of 1,2-DiOH-DBF and Robert Jack (Greifswald, Germany) for editorial help.

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