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Sphingomonas paucimobilis BPSI-3 mutant AN2 produces a red catabolite during biphenyl degradation

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The biphenyl degradation pathway of *Sphingomonas paucimobilis* BPSI-3 was investigated using a degradation-deficient mutant generated by 1-methyl-3-nitro-1-nitrosoguanidine (NTG) mutagenesis. The mutant, designated AN2, was confirmed as originating from BPSI-3 through the use of ERIC (Enterobacterial Repetitive Intergenic Consensus) PCR and by detection of the diagnostic pigment, nostoxanthin, in cellular methanol extracts. Mutant AN2 produced a yellow followed by red extracellular substance when grown in the presence of biphenyl. In the presence of 2,3-dihydroxybiphenyl, yellow followed by red then yellow compounds were formed over time. This colour change was consistent with the characteristics of a quinone, 1-phenyl-2,3-benzoquinone, which could arise from the oxidation of 2,3-dihydroxybiphenyl. A quinone was synthesised from 2,3-dihydroxybiphenyl and compared to the red compound produced by mutant AN2. Gas chromatography-mass spectrophotometry (GC-MS) confirmed that a similar quinone (4,5-dimethoxy-3-phenyl-1,2-benzoquinone) compared to the structure of the proposed biogenic compound, had been formed. This compound was also found after GC-MS analysis of mutant AN2 culture extracts. Spectrophotometric analysis of the quinone synthesised and the red product produced revealed almost identical spectral profiles. A likely inference from this evidence is that the mutant AN2 is blocked, or its activity altered, in the first gene cluster, *bphA* to *C*, of the biphenyl degradation pathway.

Keywords: biphenyl degradation; biodegradation; bioremediation; quinone

Introduction

A wide array of xenobiotic chemicals has now been dispersed throughout the environment. One class of such xenobiotics is the polychlorinated biphenyls (PCBs). It is estimated that approximately 635 million kg of PCBs were produced in the USA alone between 1929 and 1978 and that about 30% of this total is ubiquitously distributed in the environment [22,30].

Although there now exists a range of technologies with which to destroy toxic xenobiotics or remediate contaminated land [14], the trend in recent years has been towards adopting more holistic and natural approaches to the remediation of contamination in its many forms [2]. To this end, research has focused on the isolation and understanding of microorganisms capable of degrading a range of xenobiotic compounds. The first report of the biodegradation of PCBs was in the 1970s [1]. Since then, detailed knowledge has been gained on the physiochemistry, biochemistry and molecular biology involved in such degradation systems. This knowledge is necessary for effective bioremediation strategies because: (i) the catabolites produced must not exceed the toxicity of the parent compound (or be more mobile); and (ii) knowledge of the degradation pathways could identify potential bottlenecks and thus allow optimisation of biodegradation.

Sphingomonas paucimobilis BPSI-3 was previously isolated from a PCB-contaminated site in Balmain near Sydney, Australia [4,6]. This bacterium was found to have a broad substrate range, being able to attack many halogenated aromatic structures (including biphenyls, naphthalenes and benzoates [3,7]). Although much progress has been made on knowledge of the biphenyl degradation pathway in this bacterium, the initial intermediates in biphenyl degradation, such as 2,3-dihydroxybiphenyl, have never been observed under standard incubation conditions [3]. This may be due to the upper degradation pathway proceeding so rapidly that these compounds never accumulate.

To allow further study of the biphenyl degradation pathway in *S. paucimobilis* BPSI-3, we generated mutants deficient in key steps of this pathway. BPSI-3 produces a bright yellow cleavage compound, 2-hydroxy-6-oxo-6-phenylhex-2,4-dienoic acid, from biphenyl (as a consequence of 1,2-cleavage of 2,3-dihydroxybiphenyl [6]). The absence of this characteristic cleavage compound was used as a marker to select for mutants deficient in biphenyl degradation compared to the wild-type. This paper describes the production and characterisation of one of those mutants.

Materials and methods

Mutagenesis conditions using 1-methyl-3-nitro-1-nitrosoguanidine (NTG)

The mutagenesis method was modified from that of Miller [21]. An overnight culture of *S. paucimobilis* BPSI-3 [4,7] was grown in plate count broth (PC broth, 10 ml; Oxoid, Basingstoke, UK) and diluted in fresh PC broth to an absorbance of 0.1 at 600 nm. The culture was allowed to grow (25°C, 180 rpm) until an absorbance of 0.2 was

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reached. A 17-ml aliquot was taken, centrifuged (20 min, 5000 × *g*), washed twice in citrate buffer (0.1 M, pH 5.5) and resuspended in 7.5 ml of the same buffer. To 950 μl aliquots of this suspension was added 50 μl NTG (1 mg ml^{-1} in citrate buffer, Sigma Chemical Co, St Louis, MO, USA). To control tubes, 50 μl citrate buffer only was added. All suspensions were incubated at 25°C in the dark, with gentle mixing at 10-min intervals to prevent cell sedimentation.

The cell suspensions were centrifuged (5 min, 12 800 × *g*), washed three times and resuspended in 1 ml phosphate buffer (0.1 M, pH 7). A 10-fold dilution series for each sample was carried out. Dilutions of 10^{-1} to 10^{-3} (NTG-treated cells) and 10^{-4} to 10^{-6} (control cells) were spread onto plate count agar (PCA).

After a minimum of 2 days incubation (25°C, in the dark), biphenyl was added to the lids of the Petri dishes, and the incubation was continued, with the Petri dishes inverted. Within 48 h, colonies were examined for the presence of the yellow cleavage compound produced by the wild-type strain [6]. Colonies showing deviations from the wild-type were selected for further analysis.

Confirmation of origin of strain AN2 from wild-type BPSI-3

Amongst those colonies which failed to produce the yellow cleavage compound was a single colony (AN2) that produced a red compound in the presence of biphenyl. To confirm that strain AN2 derived from BPSI-3 and was not simply a contaminant, two tests were conducted.

(a) Analysis of cellular pigment: The presence of the diagnostic cellular pigment, nostoxanthin [16] was used to determine whether strain AN2 was a member of *Sphingomonas*. A loopful of cells from cultures (48 h, 25°C) grown on PCA was extracted in 1 ml HPLC grade methanol (Mallinckrodt, Sydney, Australia) by vortexing the suspension which was centrifuged (5 min, 11 000 × *g*) and the supernatant was retained. The spectrum of the cellular extract was determined over 400–650 nm using methanol to blank the spectrophotometer. *Escherichia coli* (cream colored) and *Serratia marsescens* (red) were used as negative controls.

(b) ERIC-PCR: ERIC PCR was used to confirm that strains BPSI-3 and AN2 were of the same clonal line. Cells from single colonies were streaked onto PCA plates and incubated at 25°C until growth was apparent (typically 24–48 h). Template DNA was extracted from approximately 20 mg cells of strains BPSI-3 and AN2 using the method of Gillings and Fahy [10].

PCR was carried out on the template DNA using Enterobacterial Repetitive Intergenic Consensus (ERIC) primers of the following sequences: ERIC1R—5′CACTTAGGGGTCCTCGAATGTA and ERIC2—5′AAGTAAGTGACTGGGGTGAGCG [27]. Genereleaser (9 μl, Bioventures Inc) was mixed with the target DNA (1 μl; *ca* 50 ng) in a microcentrifuge tube (0.5 ml) and overlaid with two drops of mineral oil. These tubes were heated on high for 7 min in a 750 watt microwave oven. An Erlenmeyer flask containing 100 ml water was included as a microwave sink. The tubes were incubated at 80°C in a preheated thermal cycler (Hybaid, Omni-E, Integrated Sciences, Sydney, Australia) for 10 min. Master mix (40 μl) was then added to each tube. Final concentrations of reagents in the PCR were: 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% (w/v) Triton X-100, 4 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP), 0.5 μM each primer, 20 μg ml^{-1} RNAse A and 2 U *Taq* polymerase. Dispensing of DNA, Genereleaser and master mixes was carried out with aerosol pipette tips and the master mix was made up using dedicated pipettes. Negative controls of water only and Genereleaser only were included in every round of PCR. The PCR was carried out using the following thermal cycle: 94°C, 3 min, 1 cycle; 94°C, 30 s, 52°C, 30 s, 68°C, 8 min, 35 cycles; 68°C, 15 min, 1 cycle.

PCR products were analysed using electrophoresis on 2% (w/v) agarose gels cast in TBE buffer (pH 8.3 [24]). Gels were run in TBE at 110 volts for approximately 1.5 h, stained with ethidium bromide (1 μg ml^{-1}) for 40 min and destained in TBE buffer for 30 min. Gels were visualised and photographed using transmitted UV light and Polaroid 667 film [24].

Synthesis of quinone (postulated red catabolite) for spectrophotometry

Silver nitrate (3 g) in water (4 ml) was added to sodium hydroxide (1.4 g) in water (8 ml). The precipitated silver oxide was filtered under vacuum, washed to neutrality (with water) and dried over silica gel (vacuum).

2,3-Dihydroxybiphenyl (1 mg; Wako Pure Chemical Co, Tokyo, Japan) in ethanol (10 ml) was added to silver oxide (5 mg) with stirring. An aliquot was removed immediately for gas chromatography mass spectrometry (GC-MS) analysis (see below) and after 15 min for spectral analysis (Beckman DU 640 spectrophotometer). A 1.5-ml aliquot was removed after 1 h and approximately 1 μg of potassium permanganate was added (to promote polymerisation of the quinone). This reaction was allowed to progress at room temperature and was also monitored periodically by spectrophotometry as above.

Production of red catabolite by strain AN2

Strains AN2 and BPSI-3 were grown separately on PCA and biphenyl as previously described [7] until colour production was apparent (typically 48–72 h). Cells were washed from the plate [7] and the suspension was centrifuged at 11 000 × *g* (5 min, room temperature). The supernatant was analysed by spectrophotometry as described above.

Analysis of putative quinone (red catabolite)

Strain AN2 was inoculated onto PCA with 20 mg biphenyl (provided as crystals in the lid [7]) followed by a further 20 mg after 24 h. The PCA (containing the red compound) was gently macerated and melted in a water bath (80°C). The molten agar was extracted with ethyl acetate and toluene (5:1, 250 ml) and orthophosphoric acid (0.7 ml). The organic layer was dried over anhydrous sodium sulphate and concentrated *in vacuo* to approximately 5 ml. The concentrate was centrifuged (2 min, 3000 × *g*) to remove particulate matter and the supernatant was derivatised with

diazomethane for GC-MS analysis. Diazomethane was generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, using the Diazald^{R} kit according to the manufacturer's instructions (Aldrich Chemical Company, Milwaukee, WI, USA).

GC-MS analysis—synthesised 'quinone'

GC-MS was performed on a Fison's Instruments MD800, using an SGE BPX-5 column (25 m × 0.32 mm ID and 0.25 μm film thickness). Electron ionisation was used at an electron energy of 70 eV. All other conditions were as previously described [7].

Results

S. paucimobilis BPSI-3 mutant AN2

Approximately 3000 colonies were screened for mutations in the biphenyl degradation pathway. Several putative mutants were obtained; most showed no colour evolution when grown in the presence of biphenyl. The mutant AN2 was chosen for further study based on the fact that it produced a red product only when exposed to biphenyl, thus indicating a difference in the mode of degradation compared to the wild-type. The red product was postulated to be a quinone. Interestingly, the pattern of coloured products varied over time. In the presence of 2,3-dihydroxybiphenyl for instance, yellow followed by red then yellow compounds were formed during incubation.

Confirmation of the origin of the mutant was gained from examination of the cellular methanol extracts from strains BPSI-3 and AN2, *Escherichia coli* and *Serratia marcescens* cells (Figure 1). Strains AN2 and BPSI-3 produce yellow colonies, *E. coli* is cream colored and *S. marcescens* is red/pink. The mutant AN2 and the wild-type BPSI-3 gave identical spectra, characteristic of the carotenoid nostoxanthin [16]. The other bacteria gave different spectra from each other and from strains BPSI-3 and AN2. With the use of ERIC-PCR, we confirmed that strain AN2 originated from the wild-type, as it generated a DNA fingerprint or banding pattern of PCR products identical to BPSI-3 (Figure 2). Both sets of results provide strong evidence that strain AN2 is *S. paucimobilis* and was derived from strain BPSI-3.

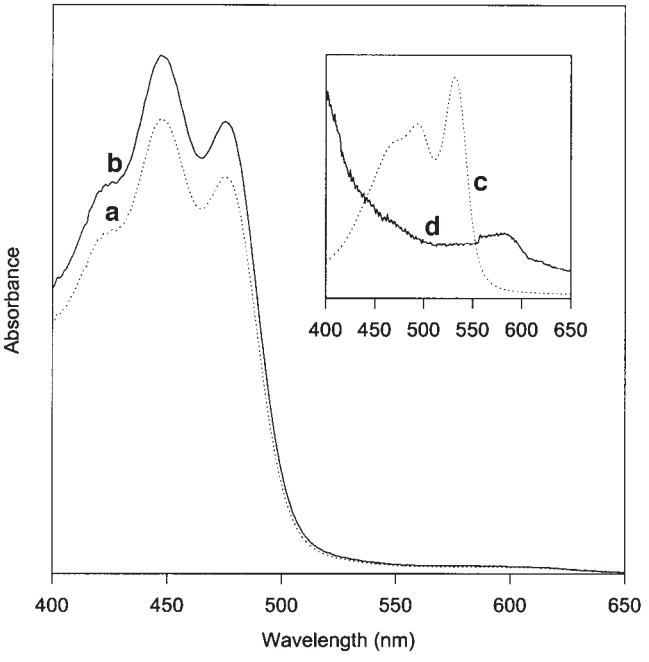

Figure 1 Spectra produced from cellular methanol extracts of *S. paucimobilis* BPSI-3 (wild type) (a), *S. paucimobilis* AN2 (b), *S. marcescens* (c) and *E. coli* (d). Only (a) and (b) exhibit spectra characteristic of the carotenoid, nostoxanthin, a diagnostic feature of *Sphingomonas (Pseudomonas) paucimobilis* [16].

GC-MS analysis of biphenyl catabolites (strain AN2)

Addition of biphenyl to a culture of strain AN2 led to production of a red pigment. GC-MS analysis of a methylated extract was dominated by uncatabolised biphenyl with only minor amounts of the catabolites previously observed from wild-type BPSI-3 [3]. A notable exception was a peak eluting at 46.06 min which had a molecular ion at 244 amu. Analysis of the M/M+1/M+2 ratios suggested a molecular formula of $C_{14}H_{12}O_4$ for this ion (Found 100:15:1.9; $C_{14}H_{12}O_4$ requires 100:15.5:1.9). The first fragmentation in the electron ionisation spectrum at 153 amu corresponds to the concomitant loss of 2 × CO, CH_3OH and CH_3O. This was followed by a loss of 28 (CO) to the base peak at 125 amu. A peak at 77 amu confirmed the presence of a phenyl group. These data were consistent with a structure of 4,5-dimethoxy-3-phenyl-1,2-benzoquinone or 2,5-dimethoxy-3-phenyl-1,4-benzoquinone for this peak. In particular, the initial loss of one mole of methanol and one methoxide is consistent with the dimethoxy arrangement. This structure was confirmed by the synthesis results.

Quinone synthesis from 2,3-dihydroxybiphenyl

Upon addition of the 2,3-dihydroxybiphenyl to methanolic silver oxide, the colour of the reaction changed from colourless to bright yellow indicating oxidation to form a

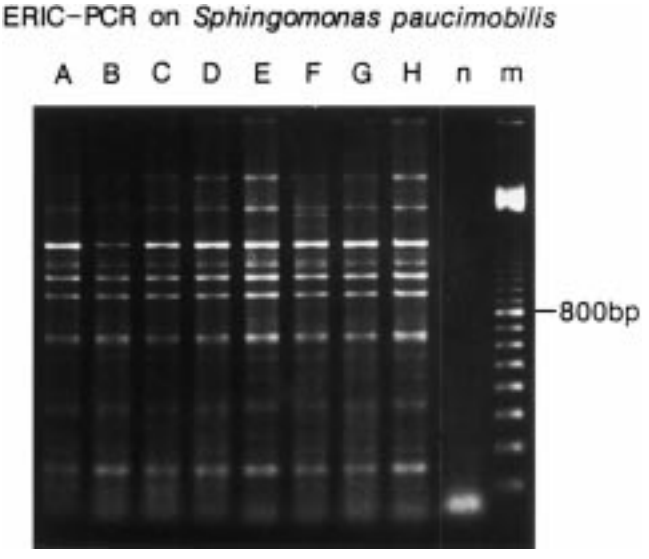

Figure 2 Electrophoretic analysis of ERIC-PCR fingerprints. Genomic DNA was amplified using ERIC-PCR [18] and electrophoresed on 2% agarose gels. Tracks are as follows (a)–(d) BPSI-3 wild-type, (e)–(h) BPSI-3/AN2 mutant, (n) negative control, (m) 100-bp ladder (Pharmacia LKB, Sydney, Australia).

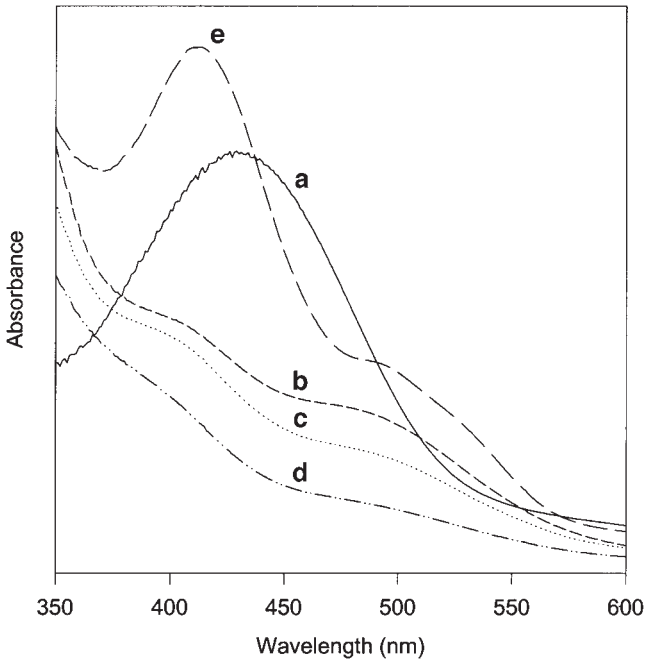

Figure 3 Formation of 1-phenyl-2,3-benzoquinone from 2,3-dihydroxybiphenyl and freshly prepared silver oxide in ethanol at room temperature after 15 min (a). A 1.5-ml aliquot of this reaction was taken and approximately 1 mg $KMnO_4$ added (to promote polymerisation). Spectra were then determined after 24 h (b), 72 h (c) and 9 days (d) at room temperature. The spectrum (e), represents the aqueous fraction from culture supernatant taken from the plate (see text for details) and mirrors the artificially 'polymerised' quinone (see text for explanation). NB: this figure is qualitative only to show changes in spectral patterns rather than the amount of product formed.

quinone. The spectrum of the product exhibited a maximum at 429 nm (Figure 3), consistent with a yellow colouration. The addition of sodium borohydride to an aliquot of this reaction yielded a colourless solution (Figure 4) indicating that the coloured compound was reducible. After exposure to a catalytic amount of potassium permanganate, the colour changed from yellow to reddish-brown over time. This was reflected in the spectrum which changed markedly, showing a shift with inflections around 400 and 490 nm rather than the defined maxima seen previously.

The mass spectrum of the synthesised quinone (electrospray) revealed a M+1 ion at m/z 273 when the synthesis was carried out in ethanol and m/z 245 in methanol. MS-MS of the 245 amu peak revealed a pattern very similar to the biogenic pigment with initial loss of 91 amu

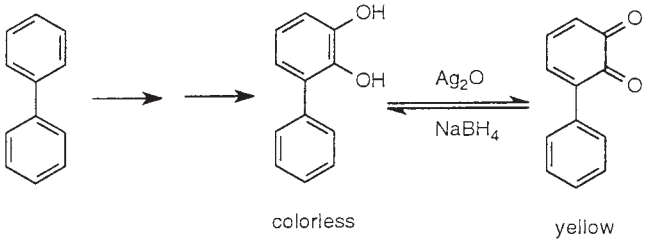

Figure 4 Redox reaction and formation between 2,3-dihydroxybiphenyl and 1-phenyl-2,3-benzoquinone. A dihydroxy compound (colourless) can be oxidised to quinone (yellow) using freshly prepared silver oxide, and the quinone reduced back to the diol using sodium borohydride.

(CH_3OH; CH_3O and $2 \times CO$) to 154 amu. In addition, there was a weak peak at 217 amu, corresponding to initial loss of CO. These data are consistent with 4,5-dimethoxy-3-phenyl -1,2-benzoquinone. This analysis was also supported by the literature [28] where oxidation of *o*-catechol with lead (IV) oxide in methanol resulted in a quantitative yield of 4,5-dimethoxy-1,2-benzoquinone. Repetition of our oxidation with silver oxide in ethanol yielded the diethoxy analogue.

Discussion

Understanding the degradation pathways of toxic chlorinated and non-chlorinated organic compounds is an essential criterion in the success of bioremediation. It is no longer possible just to monitor the disappearance of the parent substrate and proclaim biodegradation complete [25]. Furthermore, knowledge of the degradation of environmental pollutants at the molecular level may facilitate optimisation of bioremediation programs in the future. This may occur through the construction of aggressive degrading organisms and their integration into existing production processes to clean up industrial effluents. Effluent treatment in this manner could lead to sustainable industrial practices and a cleaner environment. With this in mind, we describe in this paper an investigation of the biphenyl degradation pathway in *S. paucimobilis* BPSI-3, by using mutant strains.

Mutant strain AN2 was distinguished from the wild-type strain by its ability to produce a red compound from biphenyl. This was unusual as the parent strain BPSI-3 normally produces a yellow cleavage compound during biphenyl degradation. This yellow compound is the product of *meta* cleavage of 2,3-dihydroxybiphenyl, mediated by the gene product of *bphC* [6]. Furthermore, the phenotype used to select mutant cells was unable to produce the yellow cleavage compound. Given the unusual red compound produced by strain AN2, it was important to confirm that strain AN2 originated from the wild-type and was not simply a contaminating bacterium. PCR has been shown previously in our laboratory to be a useful tool for determining molecular genetic profiles based on the ERIC primer. There is now evidence to suggest that repetitive element PCR as originally described [18,27] is actually based on annealing of primers to sites with only partial homology [11,12], and is in essence a variant of the Randomly Amplified Polymorphic DNA assay [29,31]. Thus, any pair of long primers can generate complex fingerprints, which may be then used to screen for the genetic lesion produced during the mutation process, thereby speeding the isolation and characterisation of the genes involved. We believe that this is the first report of ERIC-PCR being used as a test for confirming that a mutant was derived from a wild-type organism.

Genes in the biphenyl degradation pathway have now been well characterised and appear to be conserved in a number of Gram-positive [19] and Gram-negative organisms [9,26]. The gene product responsible for cleaving 2,3-dihydroxybiphenyl to produce the yellow cleavage compound (2-hydroxy-6-oxo-phenylhex-2,4-dienoic acid) is coded for by *bphC*. To our knowledge, red compounds have

not been described previously in the degradation of biphenyl but have been reported in the degradation of other compounds such as carbofuran. Recently, *Sphingomonas* sp strain CF06, capable of using the insecticide carbofuran as its sole source of carbon and nitrogen, was isolated from a carbofuran-contaminated soil [8]. With carbofuran as sole carbon source and strain CF06 grown in minimal medium, strain CF06 produced a red metabolite from carbofuran after 48 h which turned yellow upon prolonged incubation [8]. No explanation was made by the authors as to the identity of this metabolite. In our study, based on (i) examination of the biphenyl degradation pathway and (ii) analysis of the catabolites produced by strain AN2, the most likely compound to produce a red colour was 2,3-dihydroxybiphenyl via formation and polymerisation of 1-phenyl-2,3-benzoquinone (Figure 5) or a similar compound, possibly due to mutations in the gene/s controlling either initial hydroxylation events (products of *bphA* (complex) and *bphB*) or the ring cleavage enzyme, bphC.

The production of a quinone in the biodegradation of biphenyl by strain AN2 was suggested by UV-Vis spectrophotometry and was indicated by GC-MS of the methylated extract. The fragmentation pattern of the quinone was consistent with two structures (4,5-dimethoxy-3-phenyl-1,2-benzoquinone or 2,5-dimethoxy-3-phenyl-1,4-benzoquinone) which combine biogenic considerations (1,2 oxygenation pattern) with the necessity to have one methoxide with a β-hydrogen to explain the initial loss of one mole of methanol and one of methoxide. Synthesis of 4,5-dimethoxy-3-phenyl-1,2-benzoquinone from 2,3-dihydroxybiphenyl gave a compound with similar UV-Vis and mass spectral characteristics, supporting the presence of quinones

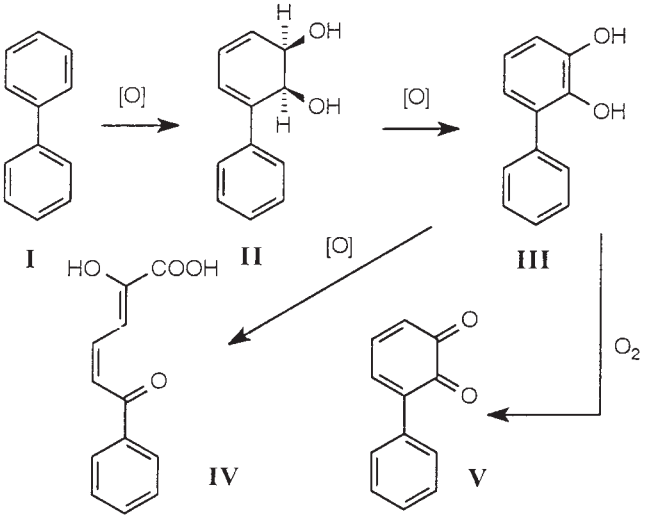

Figure 5 Possible route of quinone formation in the biphenyl degradation pathway. Compounds II–IV represent known intermediates in the degradation of biphenyl (I). A quinone (1-phenyl-2,3-benzoquinone, V) could form in equilibrium with compound III, 2,3-dihydroxybiphenyl in mutant *S. paucimobilis* BPSI-3 AN2, causing the production of the novel red compound which is different from the wild-type organism. Note that in the biodegradation of biphenyl by strain AN2, we found mass spectra consistent with the compounds 4,5-dimethoxy-3-phenyl-1,2-benzoquinone or 2,5-dimethoxy-3-phenyl-1,4-benzoquinone. Therefore, it is likely that mutant AN2 is blocked at *bphC* and may perform multiple dioxygenation of biphenyl.

in the biodegradation of biphenyl by strain AN2. To investigate this further, the quinone was chemically synthesised from commercially available 2,3-dihydroxybiphenyl and polymerisation was initiated with the addition of a catalytic amount of potassium permanganate. Spectrophotometric analysis of the synthetic and biogenic substances revealed virtually identical spectra which were consistent with some type of oxidation event in both cases.

It has been shown on numerous occasions that substrates may be catabolised to more deleterious compounds such as quinones. One such example is the bacterium *Sphingomonas chlorophenolica* RA-2 which degrades pentachlorophenol via the toxic intermediates, chlorinated hydroquinones [20]. In this case, the bacterium balances acquisition of carbon for growth from the parent compound while accumulating only low levels of the toxic chlorohydroquinones. Cellular reducing agents, such as glutathione, are thought to play an important role in maintaining the hydroquinones in their reduced form, thus reducing cellular toxicity [17,20].

In the process described above, toxicity is investigated in terms of the quinone being present in a sub-unit form (ie non-polymerised) and as a chlorinated derivative. Whether the quinone produced by strain AN2 falls into this category is questionable on two grounds. First, the structure proposed for the quinone in this study is non-chlorinated as it is generated from biphenyl, not chlorobiphenyl. The addition and stereochemistry of a chlorine substituent, on many molecules including PCBs, is known to exacerbate toxicity [13,15]. Second, polymerisation of quinones and hydroxylated compounds may result in their detoxification via humification. This process commonly occurs in soil resulting in the removal of toxic pollutants and other compounds from the aqueous soil fraction [25]. Thus, polymerisation of the quinone produced by strain AN2 could lead to the amelioration of this quinone, if indeed, it proved to be toxic. The fungal mediation of pentachlorophenol polymerisation with ferulic acid (a humic acid precursor) has recently been shown in *Phanerochaete chrysosporium* [23] and was suggested as a mechanism for its detoxification. The wild-type BPSI-3 has also been shown to produce polymerised products which were thought to arise from the polymerisation of the biphenyl catabolites, 2,3-dihydro-2,3-dihydroxybiphenyl and 2,3-dihydroxybiphenyl [5]. These polymers were brown and therefore visually quite different from those compounds produced by strain AN2, again pointing to differences in catabolism between the wild-type and mutant strain. Thus, although strain AN2 may produce only a quinone or quinone-like compounds from biphenyl, it may prove to be a useful detoxifier of toxic aromatic compounds. Further work is being conducted in our laboratory to determine the exact nature of the mutation in strain AN2 at a molecular level.

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