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5-Chloropicolinic acid is produced by specific degradation of 4-chlorobenzoic acid by *Sphingomonas paucimobilis* BPSI-3

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We have previously shown that the bacterium *Sphingomonas paucimobilis* BPSI-3, isolated from PCB-contaminated soil, can degrade halogenated biphenyls, naphthalenes, catechols and benzoic acids. However, before such an organism can be used in bioremediation, it is important to characterise the degradation products and determine the degradation pathways to ensure that compounds more toxic or mobile than the original contaminants are not produced. In the degradation of 4-chlorobiphenyl, *S. paucimobilis* BPSI-3 produces a novel chlorinated picolinic acid. In this paper, we show that 4-chlorobenzoate is an intermediate in this degradation and, through ^{15}N-labelling, that 5-chloropicolinate is the only nitrogenous metabolite isolated under the extraction conditions used. The position of the chlorine indicates that degradation of 4-chlorocatechol occurs exclusively *via* a 2,3-extradiol cleavage. These data allow us to postulate a more definitive catabolic pathway for the biodegradation of 4-chlorobiphenyl to 5-chloro-2-hydroxymuconic acid semialdehyde *via* 4-chlorobenzoate in *S. paucimobilis* BPSI-3.

Keywords: biphenyl degradation; biodegradation; bioremediation; chloropicolinic acid

Introduction

Polychlorinated biphenyls (PCBs) were first discovered to be ubiquitous environmental pollutants in the 1960s [3]. Subsequently, their manufacture has been terminated, although they still persist in many environments. Concern is growing over their toxic effects, specifically on marine mammals [31], as well as their role as possible oestrogen mimics (see [23] for example).

There are various techniques available for destruction of PCBs and PCB-containing wastes [17]. However, these will probably not be cost-effective for treatment of low concentration wastes typically found in soils and sediments. Furthermore, some technologies, such as incineration, may exacerbate pollution through the production of dioxins, compounds considered more toxic than the parent compound. Interest has now turned to the use of microorganisms for degradation of PCBs and other organochlorines (see for example [12]).

PCBs were first shown to be amenable to biodegradation by Ahmed and Focht [1]. Many microbes have now been isolated that are capable of degrading chlorinated and non-chlorinated biphenyls, generally via the intermediate chlorobenzoic acid. Some organisms, however, can exacerbate the toxicity of chlorinated biphenyls through the production of arene oxides as intermediates in the degradation process [11,30]. Furthermore, deleterious compounds, such as the antibiotic protoanemonin, are produced from other chloroaromatic compounds and are thought to contribute to their ecotoxicity [8,9,24]. Therefore, any biodegradation approaches to the remediation of contaminated sites should take into account not only the disappearance of the parent substrate, but also the appearance of catabolites. Furthermore, many microorganisms can only degrade chlorinated biphenyls to their corresponding chlorobenzoates. The first report of 4-chlorobiphenyl mineralisation to CO_2 and water *via* 4-chlorobenzoate was by Shields *et al* [29]. They found that mineralisation was mediated by the plasmid pSS50, located in several species of *Alcaligenes* and *Acinetobacter*. Exhaustive analytical procedures are therefore required not only to monitor biodegradation efficacy, but also to characterise interesting microbial isolates catabolically for their ability to degrade a variety of pollutants.

Sphingomonas paucimobilis BPSI-3 was isolated from a PCB-contaminated site in Balmain near Sydney [15]. It has a broad substrate range, being able to attack many halogenated aromatic structures including biphenyls, naphthalenes, catechols and benzoates [13,15]. In the course of these studies, *S. paucimobilis* BPSI-3 was found to produce a chloropicolinic acid from 4-chlorobiphenyl [14]. Gas chromatography-mass spectrometry (GC-MS) was used to identify this compound through comparison of the fragmentation pattern against library standards. A pathway was postulated for its formation from 4-chlorobenzoic acid based on known catabolic pathways although, from the available data, it was not possible to assign the position of the chlorine on the picolinate ring. Such stereochemical assignments of substituted muconic acid semialdehydes have previously been based on GC, GC-MS and UV characteristics of the products, but these techniques are notoriously insensitive to the identification of positional isomers.

In this paper, we describe the use of ^{15}N-labelling experiments and nuclear magnetic resonance (NMR) spectroscopy to elucidate which isomer of chloropicolinic acid is produced by *S. paucimobilis* BPSI-3 from 4-chlorobenzoic acid. These data have allowed us to propose a more detailed pathway for catabolism of 4-chlorobenzoic acid by *S. paucimobilis* BPSI-3 based on unambiguous assignment

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of the chloropicolinic acid structure and, by inference, the structure of the precursor, muconic acid semialdehyde.

Materials and methods

Bacterial culture

Cultures of *S. paucimobilis* (24–40 h; 25°C) were grown and harvested as previously described [14,15] in PCBMS, a minimal medium composed of: solution A: 2.25 g $(NH_4)_2SO_4$ in 890 ml distilled water; solution B (g L^{-1}): K_2HPO_4, 44.0; KH_2PO_4, 17.0; pH 7.0 ± 0.02; solution C (g L^{-1}): $MgSO_4 \cdot 7H_2O$, 19.5; $MnSO_4 \cdot H_2O$, 5.0; $FeSO_4 \cdot 7H_2O$, 1.0; $CaSO_4 \cdot 2H_2O$, 0.3; conc H_2SO_4, 500 μl (to prevent precipitation). Solutions A and B were autoclaved at 121°C for 15 min and solution C was filter sterilised (0.22μm). A 100-ml volume of solution B, 10 ml of solution C and the 890 ml of solution A were mixed after sterilising to make 1 L. The cell suspension was washed twice in PCBMS and added to 50 ml PCBMS (in a 250-ml Erlenmeyer flask) containing dextrose (1 g L^{-1}) and 4-chlorobenzoate (100 mg L^{-1}) to give a final absorbance at 600 nm of 0.4. The culture was incubated in the dark with shaking (25°C; 180 rpm). After 24 h, the whole culture was acidified to pH 2–3 using orthophosphoric acid (Analar, BDH, Sydney, Australia) and centrifuged (*ca* 10 000 × *g*, 30 min, 4°C) to remove cells and debris. The supernatant was extracted with two volumes of ethyl acetate (HPLC grade, BDH).

The extract was dried over anhydrous sodium sulfate and concentrated to approximately 500 μl *in vacuo* using gentle heat (30°C). The sample volume was reduced further under a stream of nitrogen and methylated with diazomethane in ether. Diazomethane was generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide using the Diazald®kit according to the manufacturer's instructions (Aldrich Chemical Company, Milwaukee, WI, USA).

^{15}N-labelling conditions

Cultures of *S. paucimobilis* BPSI-3 were produced as described previously [14,15], with some modifications. The labelling experiment was carried out using [^{15}N]-ammonium sulfate (99atom%, ICN, Aurora, OH, USA)-amended mineral salts' solution (PCBMS) [15]. The ammonium sulfate solution was filter sterilized (0.22 μm, to minimise loss of the ^{15}N label) then made up according to original methods [15]. Culture and extraction conditions were then followed as described above.

Chemical analyses

GC-MS analysis of the sample was performed on an MD800 GC-MS system (Finnigan MassLab, Manchester, UK) coupled to a Carlo-Erba GC8000 GC. The GC column was a SGE BPX5 length 25 m, internal diameter 0.22 mm, with a 0.25 μm film thickness. The injection (2 μl) was performed in the splitless mode at an injector temperature of 250°C. The column temperature was held at 40°C for 2 min then increased at 5°C min^{-1} to 280°C where it was held for 5 min. The carrier gas was helium at an inlet pressure of 70 kPa. Mass spectra were obtained in the EI mode (70 eV), the source temperature was 200°C. The mass range from 40 to 500 Da was scanned in 2 s. Possible structures were identified by comparison of the mass spectra with those of a mass spectral database (National Institute of Standards and Technology, Gaithersburg, MD, USA), previous results [14] and by analysis of fragmentation patterns.

Nuclear magnetic resonance spectroscopy

The NMR sample was prepared from the uniformly ^{15}N-labeled, methylated extract (~2 mg) by dissolving it in $CDCl_3$ (0.5 ml; 99.96 atom%, Aldrich, Milwaukee, WI, USA) and filtered into an NMR tube (Wilmald, Buena, NJ, USA, PP527). The sample was degassed and equilibrated under an atmosphere of nitrogen.

NMR data were acquired on a Bruker DMX600 (600 MHz) NMR spectrometer at 30°C and processed using xwinNMR (version 1.3; Bruker). All NMR experiments were run with quadrature detection with an ^{1}H spectral width of 6614 Hz and a recycle delay of 1 s. Chemical shifts were referenced to internal TMS (0 ppm). High power ^{1}H $\pi/2$ pulses were determined to be 7.25 μs and low power (for MLEV-17 spin lock) at 32.4 μs. MLEV sequences were flanked with trim pulses at the same low power of 2 ms duration. ^{15}N high power $\pi/2$ pulse was 39 μs and a low power pulse of 118 μs was used for GARP decoupling. Gradient pulses were delivered along the *z*-axis with a ratio of 35:15:25 of full power for selection of ^{15}N-^{1}H zero and double quantum coherence using a 1024-step Gaussian program.

Data for simple 1D experiments were acquired using a single 60° pulse with a recycle delay of 1 s. Sixty-four K real points were acquired and zero filled to 128 K and then Gaussian multiplied for resolution enhancement. ^{15}N filtered spectra were acquired using a 1 D version of the long-range zero and double quantum correlation experiment of Bax [7] but modified with gradient pulses [32] for better double quantum selection. Selection for $^2J_{NH}$ coupling was achieved using a delay of 45 ms (J 11 Hz; 32 scans) and for $^3J_{NH}$ using a delay of 500 ms (J 1 Hz; 2048 scans). No decoupling was used in t2 due to the antiphase nature of the signals at the end of the pulse sequence. FIDs (8 K data points) were processed for resolution enhancement using a strong Gaussian apodisation and zero filled to 32 K. The spectra were magnitude calculated after Fourier transformation. The same experiment (J 11 Hz; 1024 scans) was modified with a trailing TOCSY transfer [21] of 72 ms. GARP decoupling was used during t2 and the final spectrum was magnitude calculated as above.

The size of the ^{15}N–^{1}H coupling constants was measured by running the same experiment with and without decoupling during t2. When decoupling was used, an extra 1/2J delay [6] was incorporated after the last ^{15}N pulse to allow the antiphase signals to refocus. In this case, the final spectrum was not magnitude calculated but otherwise processed as above.

Proton and ^{15}N assignments were achieved using the simple proton detected, zero and double quantum coherence method of Bax [6]. No decoupling was used during the acquisition and the original 2 K data points was zero filled to 4 K in f2. One hundred and twenty-eight experiments (four scans each) in t1 (1000 ppm) were zero filled to 512 points and processed using quadrature filtering.

Results and discussion

We have previously shown [14] that *S. paucimobilis* can degrade 4-chlorobiphenyl to produce chlorohydroxymuconic acid semialdehyde, 4-chlorophenol and many other metabolites, one of which was consistent with a chloropicolinic acid. The picolinic acid was not formed in sterile controls or if nitrogen was omitted from the medium. We repeated the previous experiments with 4-chlorobenzoic acid and extracting the metabolites with acidified ethyl acetate yielded a pale oil, which was methylated (CH_2N_2/ether) to facilitate analysis by GC-MS (Figure 1). 4-Chlorophenol (**1**) was the major metabolite, along with chlorohydroxymuconic acid semialdehyde (**3**) {methyl ester} and chloropicolinic acid (**2**) {methyl ester} confirming that 4-chlorobenzoic acid is an intermediate in the biodegradation of 4-chlorobiphenyl by *S. paucimobilis* BPSI-3. This conclusion is based on the fact that *S paucimobilis* BPSI-3 can degrade 4-chlorobenzoic acid and that we have previously isolated the same compounds starting from 4-chlorobiphenyl [14]. Two peaks were seen for chloro-2-methoxymuconic acid semialdehyde (**3a**, **3b**) {methyl ester} which arise (presumably) from *cis-/trans*-isomers about the 2,3-double bond, trapped by methylation of the enol form. In contrast, only one peak was seen for the non-methylated metabolite (**3**) {methyl ester} indicating that enolisation occurs rapidly, but slowly enough to cause broadening of the GC peak. The observed products are generally dead-end metabolites but the production of 4-chlorophenol (**1**) was indicative of initial 1,2-dioxygenation of 4-chlorobenzoic acid, the product of which could readily lose CO_2 and water to yield **1** (Figure 2).

The peak eluting at 22.8 min (Figure 1) had a mass spectral fragmentation pattern (Figure 3a) very similar to 6-chloropicolinic acid methyl ester (National Institute of Standards and Technology). A molecular ion at *m/z* 171/173 (3:1) suggested the presence of one chlorine and one nitrogen (odd molecular weight). The peaks at *m/z* 140/142 ($M^{+\cdot} - OCH_3$) and *m/z* 112/114 ($M^{+\cdot}$–$COOCH_3$) confirmed the presence of a carbomethoxy group. Loss of HCl from the peak at 112 yielded a peak at 76 Da, consistent with a pyridine nucleus. The position of the carbomethoxy group was deduced from biogenic considerations (Figure 2) and confirmed by peaks in the mass spectrum at *m/z* 141/143 and 115/113 resulting from loss of OCH_3 and $COOCH_3$ respectively with concomitant proton transfer to the nitrogen. This can occur only when the carbomethoxy is in the 2-position. It was not possible to determine the position of the chlorine from mass spectral data.

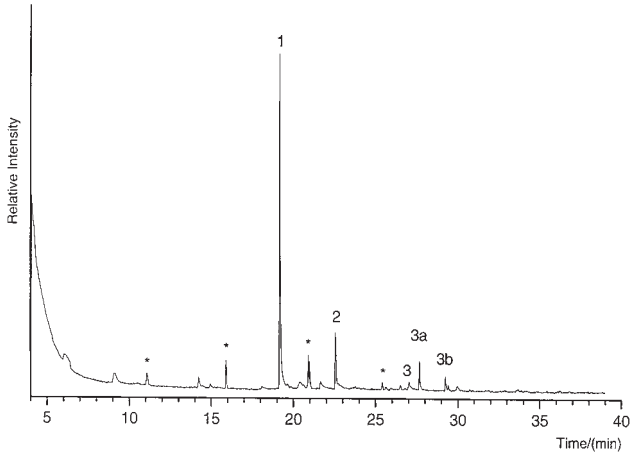

Figure 1 Total ion chromatogram of the ethyl acetate extract from *S. paucimobilis* incubated for 24 h with [^{15}N]-PCBMS medium and 4-chlorobenzoate. Prominent metabolites are 4-chlorophenol **(1)**, chloropicolinic acid **(2)** methyl ester, 5-chloro-2-hydroxymuconic acid semialdehyde **(3)** methyl ester and *cis/trans*-5-chloro-2-methoxymuconic acid semialdehyde methyl ester **(3a,3b)**. Peaks labelled with an * correspond to column bleed.

Determining the position of the chlorine substituent is not trivial, but it can be done by NMR spectroscopy. In particular, heteronuclear (^{13}C–^{1}H) long range coupling constants (HMBC) can be used. However, in this case, there were a number of factors precluding this approach. Most importantly, we wanted to identify all nitrogenous metabolites of 4-chlorobenzoate. This necessitated working with the crude extract, as any purification might remove minor metabolites or by-products. The background signals from the crude extract (eg see Figure 4a) would make interpretation of any HMBC experiment impossible. In addition, the tedious purification of the metabolities by either GC or HPLC would yield small amounts (<1 mg) of metabolites which would make the HMBC experiment difficult as its sensitivity is very low. To solve these problems, we introduced an ^{15}N label which, like ^{13}C, can be used to differentiate the isomers through chemical shift and coupling patterns, but is free from background signals.

Incubations of *S. paucimobilis* in ^{15}N PCBMS with 4-chlorobenzoate were essentially identical with those in normal PCBMS with the exception of the fragmentation pattern of the peak eluting at 22.8 min (Figure 3b). The shifting of all peaks to a heavier mass by 1 Da confirmed the presence of one N atom in the methyl ester of **2** (Figure 1) and the near-quantitative incorporation of ^{15}N. Zero and double quantum NMR experiments on the crude extract were used to establish the position of the chlorine.

According to Kemp [19], the ^{15}N chemical shift in pyridines is around 320 ppm displaying $^{2}J_{NH}$ values around 12 Hz, $^{3}J_{NH}$ around 1.5 Hz and $^{4}J_{NH}$ values of 0.2 Hz [22]. In addition, we would expect $^{3}J_{HH}$ couplings of around 8 Hz, $^{4}J_{HH}$ of around 2.5 Hz and $^{5}J_{NH}$ <1 Hz. This coupling pattern should make it possible to elucidate the structure of the picolinate, irrespective of the substitution pattern. We first used a 2 D experiment to correlate ^{15}N to ^{1}H through heteronuclear long-range coupling. This revealed one ^{15}N signal in the expected ^{15}N chemical shift range, which was coupled to two proton signals at δ 8.70 and 8.10. No other signals were observed, suggesting that there was only one constitutional isomer of methyl chloropicolinate in our mixture. In addition, there were two weak signals at δ 6.69 and 6.52, coupled to one ^{15}N signal at 123 ppm. The ^{15}N and ^{1}H chemical shifts are typical of amides. A 1 D version of the 2 D experiment (Figure 4b) optimised for a coupling constant of 12 Hz revealed only one signal (δ_H 8.70). The combination of chemical shifts (δ_H/δ_N) and coupling constant (11.0 Hz) identified the proton as H6 of a pyridine ring. Repetition of this experiment with inclusion of a refocussing delay after the last ^{15}N pulse and decoupling (GARP) of ^{15}N during the acquisition period (data not

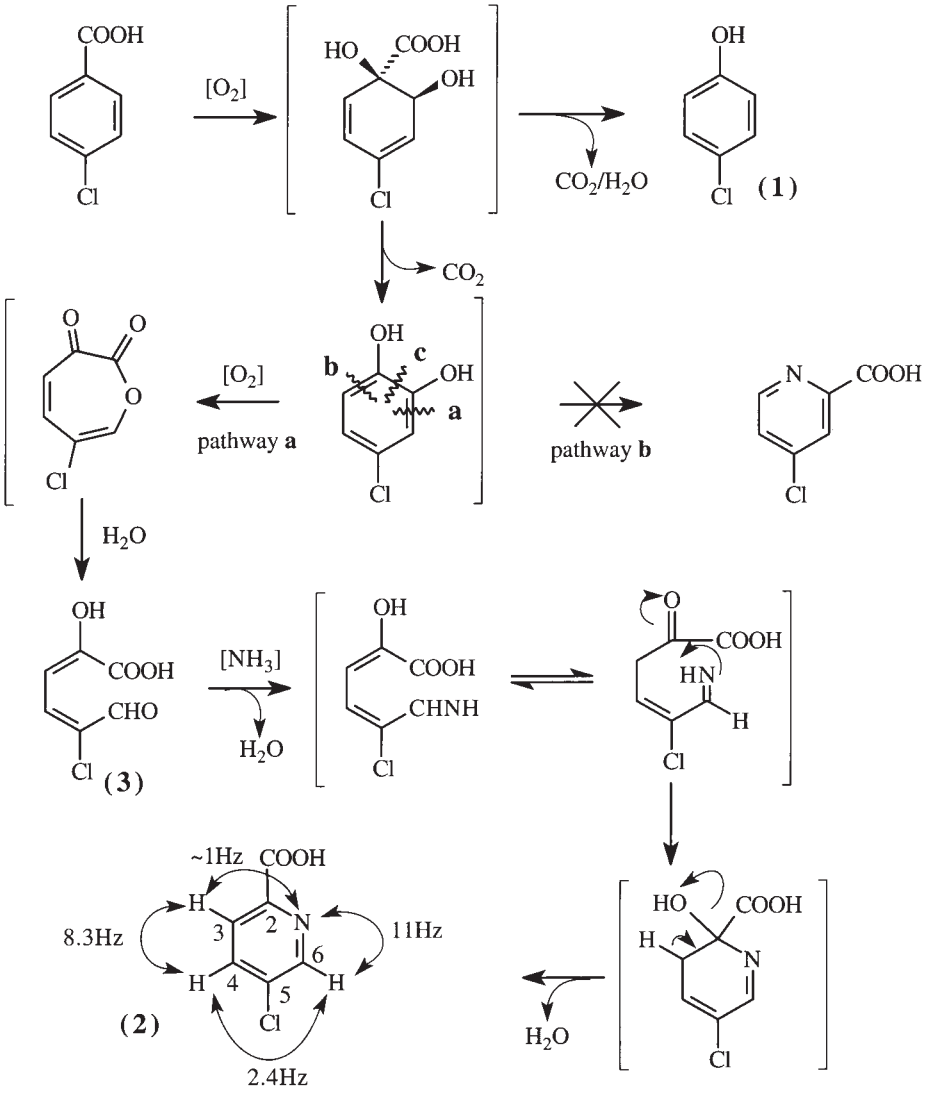

Figure 2 Catabolic pathway for the degradation of 4-chlorobenzoate to 5-chloropicolinate. Decarboxylation and *meta*-cleavage are facilitated by dioxygenases [O_2]. Three possible modes of aromatic ring cleavage are possible: proximal extradiol (a); distal extradiol (b); or intradiol cleavage. Only pathway 'a', which involves cleavage of the 2–3 bond of 4-chlorocatechol, is found in *S. paucimobilis* BPSI-3. Compounds indicated in square brackets were not isolated but are postulated intermediates. The structure of **(2)** indicates the coupling constants measured from Figure 4a and the numbering system used in the text. Structures in square brackets were not identified but inferred as intermediates on biogenic grounds.

shown) collapsed the ^{1}H signal into a doublet (J_{HH} 2.4 Hz). Thus the larger coupling (11.0 Hz) observed in the ^{1}H NMR spectrum (Figure 4a) is due to the adjacent ^{15}N and this proton (H6) has no adjacent proton. A zero/double quantum 1 D experiment optimised for a J_{HN} coupling of 1 Hz (Figure 4c) revealed a new signal at δ 8.10, a doublet (J_{HH} 8.3 Hz; Figure 4a). The third proton in the spin system was revealed by appending a TOCSY period after the last ^{15}N pulse of the refocussed zero and double quantum correlation experiment (Figure 4d), This spectrum contained the last two signals plus another at δ 7.84, which apparently had no coupling to ^{15}N but was coupled to the protons resonating at 8.10 ppm (J_{HH} 8.4 Hz) and 8.70 ppm (J_{HH} 2.4 Hz). This pattern is consistent only with 5-chloropicolinic acid (Figure 2) where the signals at 8.10 and 7.84 ppm are *ortho* to each other (J_{HH} 8.3 Hz) and H6, adjacent to the ^{15}N (J_{HN} 11.0 Hz) is separated from H4 (δ 7.84) by the chlorine at C5.

Nitrogen-containing heterocyclic compounds have previously been observed in the degradation of biphenyls. For example, Ahmed [2], postulated the presence of a 4-chlorophenyl-substituted picolinic acid as a degradation product of 4-chlorobiphenyl by some strains of *Pseudomonas putida* carrying cloned genes from *P. testosteroni* B-356. However, the picolinate still contained the chlorinated ring, and the substitution pattern was not elucidated. 2-Picolinic acid has also been reported as a metabolite of catechol [10] degradation and through the action of metapyrocatechase on 4-substituted catechols [25] and aminophenols [20]. In our case, the results are significant because the presence of 5-chloro-2-picolinate, aside from being a novel metabolite, demonstrates that *S. paucimobilis* BPSI-3 is capable of sequentially attacking the aromatic rings of 4-chlorobiphenyl—even the halogenated ring can, at least, be cleaved. In addition, the presence of the chlorine has, through ^{15}N labelling, allowed us to define unambiguously the cleavage

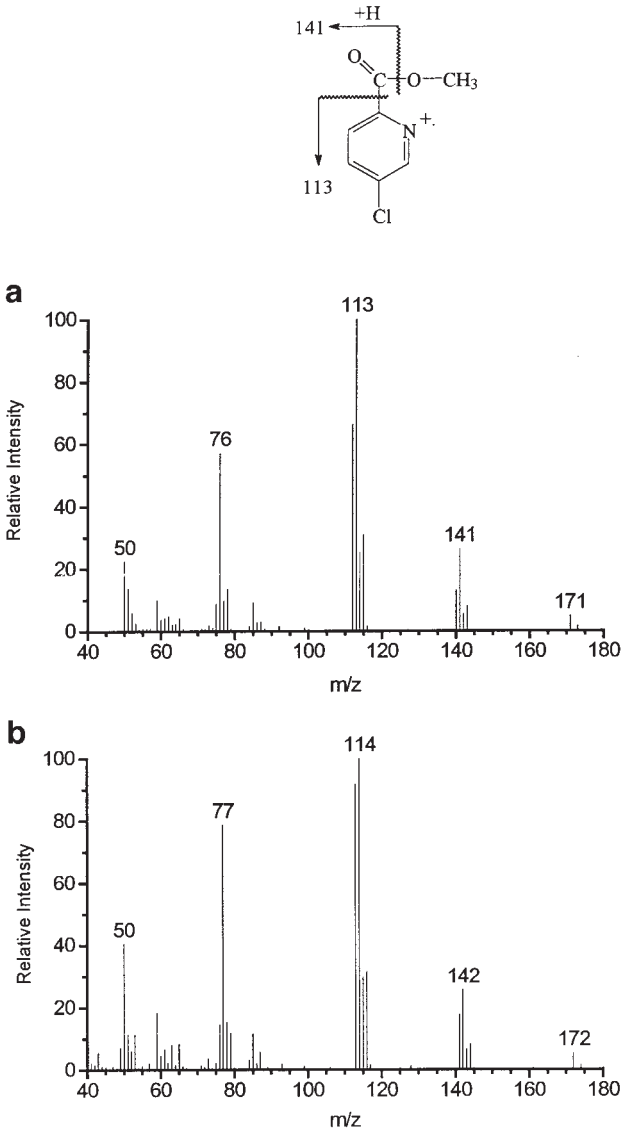

Figure 3 Electron impact (70 eV) mass spectral fragmentation pattern of unlabelled (a) and [^{15}N]-chloropicolinic acid methyl ester (b) obtained through GCMS of the methylated extract. All the isomers of chloropicolinate have very similar fragmentation patterns making them impossible to differentiate by mass spectrometry alone.

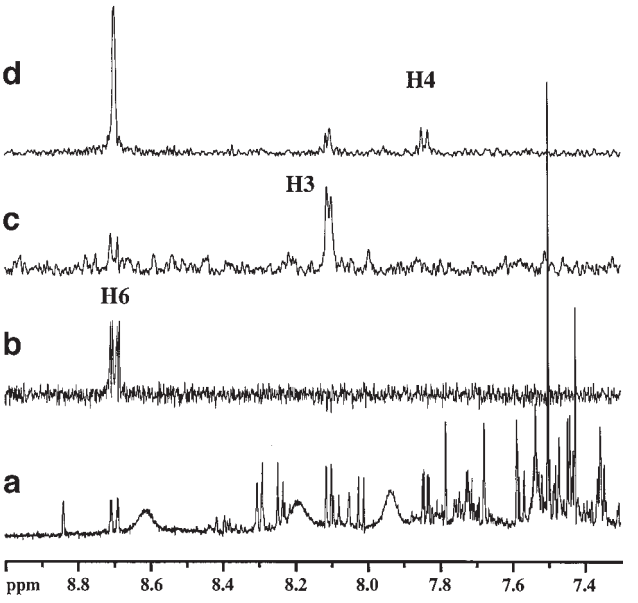

Figure 4 ^{1}H NMR spectra of the ^{15}N-labelled extract showing the normal proton NMR spectrum (a) of the crude extract, the zero and double quantum filtered spectrum optimised for a coupling of 11 Hz (b) cleanly reveals only H6 of the chloropicolinate (methyl ester). Trace (c) is optimised for a coupling of 1 Hz and shows H6 and H3. Finally, trace (d) is the same as (b) but with a trailling TOCSY transfer, showing all three protons on the pyridine ring.

pathway from benzoic acid to 2-hydroxymuconic acid semialdehyde. Previous authors [28] have inferred this pathway but assignment of muconic acid isomers through GC, GC-MS or UV spectroscopy can be unreliable as isomers that differ only in the position of the halogen are not easily differentiated.

Picolinic acid production has been attributed to the abiotic reaction of 2-hydroxymuconic acid semialdehydes with ammonia [5,27]. However, *E. coli* HB107/pSLMK1, cloned with *Pseudomonas* catechol 2,3-dioxygenase (metapyrocatechase), did not produce picolinic acids under culture conditions but these were formed when concentrated ammonia (*ca* 5 M) was added and allowed to react for 6 days [5]. In contrast, we observed relatively high yields of picolinic acids under physiological conditions with no added ammonia outside that used in the PCBMS medium, leaving open the possibility of enzyme-assisted formation of picolinates in *S. paucimobilis* BPSI-3. In other cases [4,20], the nitrogen is already present in the degradation of amino or nitro benzenes, which produce 2-aminomuconic acid semialdehydes and there is little doubt that the cyclisation and dehydration are spontaneous.

The environmental impact of releasing picolinic acids is unknown but deserves further research before organisms capable of PCB degradation are released, particularly as picolinic acids are known as dopamine β-hydroxylase inhibitors [26]. It is also important to note that although *S. paucimobilis* BPSI-3 may not be an efficient degrader of chlorobiphenyl in terms of mineralisation, it may prove to be a useful member of a bacterial consortium [16] that can achieve complete degradation of this and other organochlorines.

The data presented here show that 5-chloropicolinate is the only alkaloid produced by *S. paucimobilis* BPSI-3 from the degradation of 4-chorobiphenyl via 4-chlorobenzoic acid that can be extracted with ethyl acetate under acidic conditions. This degradation entails exclusive cleavage of the 2,3-bond in 4-chlorocatechol by the putative dioxygenase (pathway 'a', Figure 2). The resulting lactone (Criegee rearrangement [27]) would be easily hydrolysed to 5-chloro-2-hydroxymuconic acid semialdehyde, which was also observed (Figure 1), consistent only with a proximal extradiol cleavage pathway.

Acknowledgements

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Note added in proof

Since submission of this manuscript, Katsivela *et al* [18] reported the production of 5-chloropicolinic acid from 4-chlorocatechol through a similar proximal extradiol cleavage pathway by a new bacterium (LW1). The authors used our [14] mass spectral arguments to propose a structure for the chloropicolinate and confirmed this using a ^{1}H–^{13}C HMBC NMR experiment. This approach has the disadvantages that the metabolite must be purified before analysis and that relatively large quantities are required to obtain long-range ^{1}H–^{13}C correlations at natural abundance.

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