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ORIGINAL PAPER

# Aerobic biotransformation of 3-methylindole to ring cleavage products by *Cupriavidus* sp. strain KK10

**Kimiko Fukuoka · Yasuhiro Ozeki · Robert A. Kanaly**

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**Abstract** 3-Methylindole, also referred to as skatole, is a pollutant of environmental concern due to its persistence, mobility and potential health impacts. Petroleum refining, intensive livestock production and application of biosolids to agricultural lands result in releases of 3-methylindole to the environment. Even so, little is known about the aerobic biodegradation of 3-methylindole and comprehensive biotransformation pathways have not been established. Using glycerol as feedstock, the soil bacterium *Cupriavidus* sp. strain KK10 biodegraded 100 mg/L of 3-methylindole in 24 h. Cometabolic 3-methylindole biodegradation was confirmed by the identification of biotransformation products through liquid chromatography electrospray ionization tandem mass spectrometry analyses. In all, 14 3-methylindole biotransformation products were identified which revealed that biotransformation occurred through different pathways that included carbocyclic aromatic ring-fission of 3-methylindole to single-ring pyrrole carboxylic acids. This work provides first comprehensive evidence for the aerobic biotransformation mechanisms of 3-methylindole by a soil bacterium and expands our understanding of the biodegradative capabilities of members of the genus *Cupriavidus* towards heteroaromatic pollutants.

**Keywords** *Cupriavidus* · Skatole · 3-methylindole · Glycerol · Biotransformation · LC/ESI–MS/MS

## Introduction

Petroleum processing, intensive livestock production and application of biosolids to agricultural lands result in the release of nitrogen-containing organic pollutants to water and soil ecosystems and there is much interest to control and mitigate the effects of these compounds (Beier et al. 2009; Trabue et al. 2011; Cook et al. 2010; Ducey and Hunt 2013; Yan et al. 2013; Zhang et al. 2013). 3-Methylindole, also referred to as skatole, is a malodorous *N*-heterocyclic aromatic pollutant that occurs at mg/kg levels in biosolids and solid manures and mg/L levels in liquid manures (Yasuhara 1987; Schüssler and Nitschke 1999; Wu et al. 1999; De la Torre et al. 2000; Yager et al. 2014). In impacted environments, concentrations of 3-methylindole have been measured at 0.3 ug/L in surface waters and in the parts per million range in soils (Botalova and Schwarzbauer 2011; Yager et al. 2014). 3-Methylindole is considered to be a contaminant of emerging concern (CEC) partly due to its application to farmland in biosolids, where, after

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biosolids application, it persists in soil even among complex soil microbiota and is sufficiently mobile so as to be vertically transported into the soil column (Diamond, et al. 2011; Yager et al. 2014).

The biodegradation mechanisms of 3-methylindole by bacteria are not well understood and this appears to be due in part to the toxicity of this compound towards microorganisms. 3-Methylindole is described as having fairly broad bacteriostatic effects, has been shown to disrupt bacterial biofilm development, has been implicated as a potential inducer of bacterial oxidative stress, and may be genotoxic through formation of DNA adducts (Yokoyama and Carlson 1979; Deslandes et al. 2001; Regal et al. 2001; Choi et al. 2013). Indeed, there are few reports of bacterial isolates with the ability to biodegrade 3-methylindole (Fujioka and Wada, 1968; Yin et al. 2006; Yin and Gu 2006; Sharma et al. 2014). At the same time, comprehensive biotransformation pathways have not been established. Two 3-methylindole metabolites, indoline-3-carboxylic acid and indoline-3-ol were proposed through the pioneering work of Yin and Gu (2006) based upon the values of two deprotonated molecules detected in sample extracts after incubation of 3-methylindole with a *Pseudomonas* sp. isolated from mangrove sediments. Based upon these results a pathway through oxidation of the methyl group of 3-methylindole was proposed (Yin and Gu 2006). Anaerobic biodegradation of 3-methylindole by microbial consortia and sediments to carbon dioxide has been reported to occur, however downstream ring fission biotransformation products were not directly identified and only one metabolite, 3-methyloxindole has been reported (Gu and Berry 1991, 1992; Gu et al. 2002).

Overall, there are knowledge gaps in understanding what types of microorganisms may aerobically biodegrade 3-methylindole, the conditions under which 3-methylindole may be biodegraded, and the biotransformation pathway(s) that may be involved. In this work, a recently isolated soil bacterium that grew on glycerol as a sole source of carbon and energy was investigated for its abilities to cometabolically degrade 3-methylindole. This organism belonged to the genus *Cupriavidus* and rapidly depleted 3-methylindole from culture media. 3-Methylindole biodegradation was confirmed by identification of multiple biotransformation products that included aromatic ring-fission products and these results allowed for the construction of a detailed aerobic biotransformation pathway for the first time. Based upon these results, *Cupriavidus* sp. strain KK10 may be a promising candidate for further study in regard to understanding and optimizing 3-methylindole biodegradation.

## Methods

### Chemicals

3-Methylindole (98 % purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycerol (99 % purity), *N*,*N*-dimethylformamide (DMF, >99.5 % purity), salicylic acid (>99 % purity), indigo (95 % purity), pyrene (98 % purity) and organic solvents methanol and ethyl acetate (HPLC grade or higher), were purchased from Wako Chemical (Osaka, Japan). Indirubin (>98 % purity) and isatin (>98 % purity) were purchased from Tokyo Chemical Industries (Tokyo, Japan).

### Environmental isolate

Strain KK10 was isolated from a soil bacterial consortium that grew on diesel fuel (Kanaly et al. 1997, 2000; Kanaly and Watanabe 2004). The 16S rRNA gene sequence of strain KK10 showed that it was a member of the genus *Cupriavidu*s, Genbank accession number KJ123723, and details of its isolation are given in Fukuoka et al. (2015). The strain was maintained on 100 mM glycerol in Stanier's Basal Medium (SBM; Atlas 1993) by continuous rotary shaking at 28 °C in the dark.

### Quantitative analysis of 3-methylindole biodegradation and growth monitoring of strain KK10

Strain KK10 was grown on 100 mM glycerol in 20 mL of SBM in a 100-mL size conical flask to mid-log phase by rotary shaking at 28 °C in the dark, after which cells were harvested by centrifugation (8700×*g*, 10 min, 4 °C). Cell pellets were resuspended in 30 mL of phosphate buffer (50 mM, pH 7) and followed by four cell washing and centrifugation steps at 5700×*g*, 4 °C for 10 min, 8 min, 8 min and 8 min each. After the final washing step, cells were resuspended in SBM and inoculated into 15-mL volume glass culture tubes where the total volume of

SBM was 5 mL. The optical density of strain KK10 cells in the culture tubes was adjusted to OD = 1.4 after determining absorbance at 620 nm by using a V-530 UV/VIS spectrophotometer (Jasco, Tokyo, Japan). Sterile glycerol was prepared in sterile SBM at a ratio of 30:70 and aseptically transferred to culture tubes to yield a concentration of 5 mM glycerol at the start of the experiments. 3-Methylindole was solubilized in DMF in a glass vial followed by rigorous mixing until crystals were dissolved and was aseptically transferred to culture tubes to achieve a final concentration of 100 mg/L (DMF, 0.1 % *v/v*). Cultures that consisted of 3-methylindole without cells served as abiotic controls. Cultures were prepared in duplicate and incubated by reciprocal shaking at 28 °C at 150 rpm in the dark. Whole tube extractions were conducted with ethyl acetate and pyrene in ethyl acetate was utilized as the extraction standard. Organic and aqueous phases were separated and analyzed by LC with UV detection at 254 nm using a Jasco system (Tokyo, Japan) that consisted of a PU-2089 quaternary pump inline with a UV-2075 Plus UV detector. Extracts were eluted isocratically in 77 % methanol/water and separated on a Crestpak C18S 150 × 4.6 mm column (Jasco). The flow rate was 0.3 mL/min and sample injection was conducted by a Jasco AS-2057 Plus autoinjector.

Growth monitoring of strain KK10 was conducted when it was incubated with 100 mM glycerol or 100 mg/L 3-methylindole prepared in SBM as described above. Biotic controls consisted of strain KK10 cells without a carbon substrate. Culture volumes were 20 mL in 100-mL size Erlenmyer flasks that were prepared in triplicate. Cell densities in flasks were adjusted to $OD_{620} = 0.03$. All cultures were incubated by rotary shaking in the dark at 28 °C and sampling was conducted every 24 h to determine mid-log phase. At the time of sampling, culture fluids were transferred to a quartz cuvette in 600 µL aliquots and analyzed by spectrophotometer by monitoring absorbance at an optical density of 620 nm as described above.

## 3-Methylindole biotransformation assays

Strain KK10 cells were grown on glycerol and exposed to 100 mg/L 3-methylindole as described above. Cultures that consisted of 3-methylindole without strain KK10 cells and strain KK10 cells without 3-methylindole served as abiotic and biotic controls respectively. During incubation, cultures were extracted at neutral and acidic pH with ethyl acetate. Organic and aqueous phases were separated in glass separatory funnels, passed through anhydrous sodium sulfate that was prepared by overnight drying at 50 °C and extracted a second time. Sample extracts were gently heated in an aluminum heating block (Taitec, Japan) at 28 °C, and ethyl acetate was evaporated under nitrogen gas. Finally, residues were resuspended in methanol and passed through 0.45 µm PTFE syringe filters (Toyo Roshi, Tokyo) for LC–MS/MS analyses.

## Analyses of 3-methylindole biotransformation products and authentic standards by LC–UV/ESI(−)–MS/(MS)

Neutral and acidified sample extracts were analyzed separately by LC–UV/ESI(−)–MS in full scan mode using a Waters 2690 Separations Module delivery system inline with a Shimadzu SPD-10A UV–VIS detector that was interfaced with a Quattro Ultima triple stage quadrupole mass spectrometer (Micromass, Manchester, UK). Sample extracts were eluted isocratically in 77 % methanol and 23 % water at a flow rate of 0.3 mL/min through an XSelect CSH C18 column (4.6 mm I.D. × 150 mm) that was in line with a Security Guard Cartridge System pre-column fitted with a widepore C18 cartridge (Phenomenex, Torrance, CA, USA). Full scan analyses were conducted over a range of 50 to 500 *m/z* in electrospray negative ionization mode. Nitrogen was used as the nebulizing gas, the ion source temperature was 130 °C, the desolvation temperature was 350 °C, and the cone voltage was operated at 40 V. Nitrogen gas was also used as the desolvation gas (600 L/h) and the cone gas (60 L/h).

Results of the full scan analyses were examined to determine putative mass ions of interest by comparing the results from analyses of extracts from 3-methylindole biotransformation cultures with the results from analyses of extracts from abiotic and biotic controls. After selection of putative mass ions of interest, sample extract analyses were repeated by LC/ESI(−)–MS/MS by using collision induced dissociation (CID) product ion and precursor ion scan modes under mass conditions similar to as described above. For tandem mass analyses, argon gas was used as the collision cell gas and collision cell energies were employed over a

range of 3–40 eV depending upon the target analyte. The mass spectral fragmentation patterns that resulted from product ion and precursor ion scan analyses were analyzed to aid in the determination of the molecular structures of unknown 3-methylindole biotransformation products.

Authentic standards of isatin, salicylic acid, indirubin and indigo were prepared in methanol and analyzed by product ion scan analyses similarly to as described above.

### Analysis of 1H-pyrrole-2-carboxylic acid biodegradation

1*H*-pyrrole-2-carboxylic acid (>98 % purity; Tokyo Chemical Industries) biodegradation was investigated in assays that were conducted under identical conditions as described for 3-methylindole except that it was applied at a concentration of 200 mg/L.

## Results

### Quantification of 3-methylindole biodegradation by strain KK10 and growth assay

Strain KK10 cells that were growing on glycerol were exposed to 3-methylindole in varying amounts up to a relatively high concentration of 50 mg/L. Even so, 3-methylindole was completely depleted from culture media within 15 h (data not shown). Doubling the 3-methylindole concentration to 100 mg/L also had little effect on its rapid rate of biodegradation by strain KK10 as shown in Fig. 1. Compared to abiotic controls, approximately half of 100 mg/L 3-methylindole was biodegraded in 6 h. By 24 h, greater than 99 % of 3-methylindole was biodegraded which corresponded to less than 1 mg/L 3-methylindole remaining in culture media.

Strain KK10 grew well on glycerol as a sole source of carbon and energy. When incubated with 100 mM glycerol, mid-log phase occurred within 36 h and average absorbance values ($OD_{620}$) for triplicate flasks reached approximately 2.5 in 48 h when the starting cell density was equal to 0.03 (Supplementary Fig. S1). Strain KK10 was unable to utilize 3-methylindole as a carbon source for growth at this concentration (Supplementary Fig. S1).

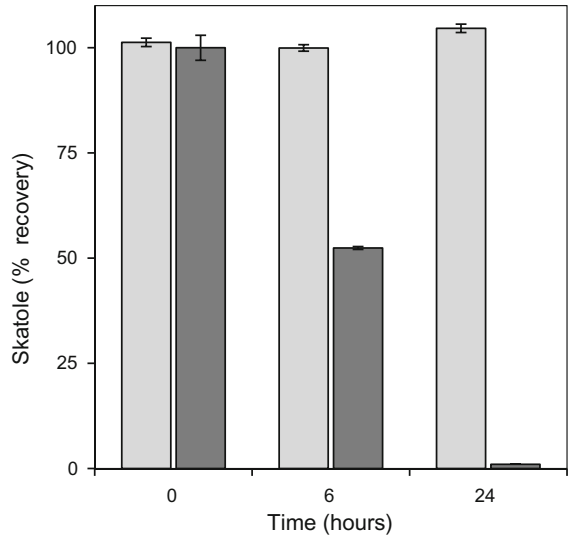


**Fig. 1** Recoveries of 3-methylindole from whole-flask extraction of cultures after 0, 6 and 24 h of incubation. Abiotic controls that consisted of 3-methylindole without cells (*white bars*); Strain KK10 cells plus 100 mg/L 3-methylindole (*gray bars*). Results are average recoveries of duplicate cultures each and the error bars represent the range

### Initial oxidation of 3-methylindole reveals multiple biotransformation pathways

$UV_{254nm}$-detectable biotransformation products of 3-methylindole by strain KK10 were separated and detected in organic extracts. The dynamics of biotransformation are shown in Fig. 2a–c. Within 6 h (Fig. 2a) multiple biotransformation products were detected and 3-methylindole was still present in the culture medium. As shown in Fig. 2b, after 24 h of exposure to 3-methylindole, at least fouteen products were detected and they are labeled as I through XIV based upon their retention times in the order that they are discussed in this report. At the same time, 3-methylindole was degraded to undetectable levels. By 48 h (Fig. 2c), biotransformation product peak size and number had decreased. These analyses confirmed rapid biotransformation of 3-methylindole and showed transient production of biotransformation products between 6 and 48 h. Because there did not appear to be large accumulations of low molar mass biotransformation products, such as IX, X and XI, pyrrole ring fission may have occurred.

Numerous large and small peaks that represented biotransformation products of 3-methylindole were

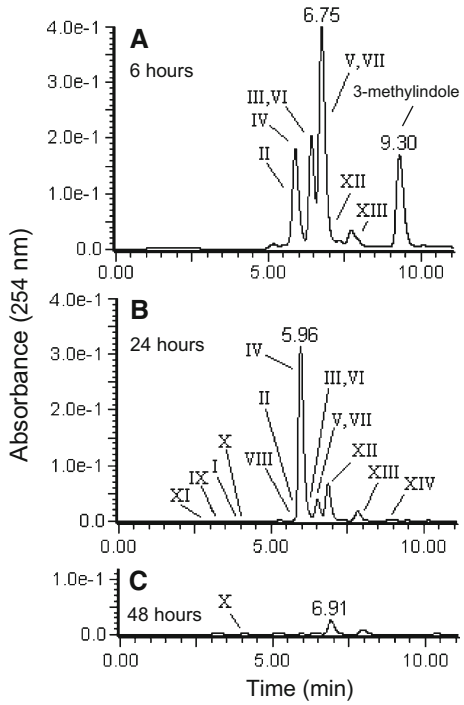


**Fig. 2** Results of LC-$UV_{254nm}$ analyses of extracts from cultures that consisted of strain KK10 cells plus 3-methylindole, **a** after 6 h; **b** after 24 h; **c** and after 48 h. 3-Methylindole biotransformation products discussed in the text are labeled I–XIV

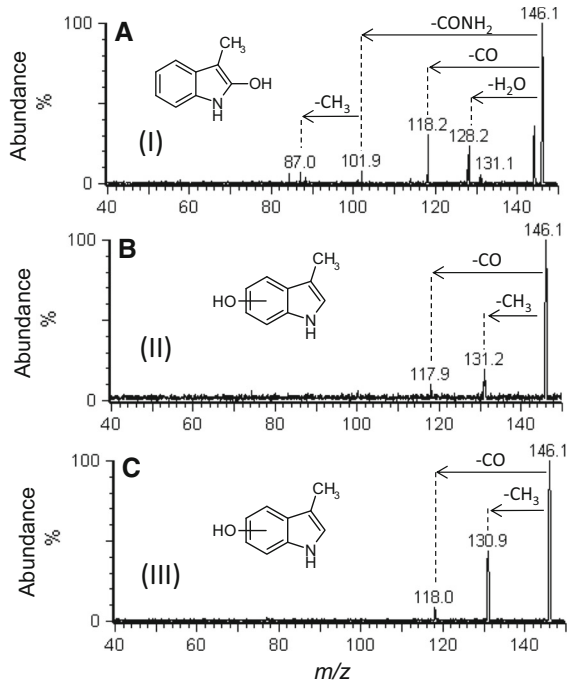


**Fig. 3** ESI(−)–MS/MS spectra acquired by product ion scan analyses of three singly oxidized 3-methylindole biotransformation products that corresponded to $[M-H]^- = 146$. **a** Product I, $t_R = 3.9$ min; **b** Product II, $t_R = 5.8$ min; **c** Product III, $t_R = 6.3$ min. The chemical structures proposed for these biotransformation products are also shown

detected by UV and mass analyses. The smallest biotransformation products by molar mass all corresponded to the deprotonated molecules, $[M-H]^- = 146$, and based upon this value they appeared to be singly oxidized products of 3-methylindole. Three products were detected at retention times ($t_R$) equal to 3.9, 5.8 and 6.3 min (products I, II and III respectively). Product ion scan analyses of product I revealed five fragmentation ions at *m/z* 131, *m/z* 128, *m/z* 118, *m/z* 102, and *m/z* 87 which each indicated losses of 15 Da ($CH_3$), 18 Da ($H_2O$), 28 Da (CO), 44 Da ($CONH_2$), and 59 Da ($CONH_2$ plus $CH_3$) respectively from the parent deprotonated molecule (Fig. 3a). Loss of water and CO from the parent deprotonated molecule supported that oxidation of the 3-methylindole molecule had occurred at an aromatic carbon atom and not on the methyl group. This conclusion was further supported by the detection of *m/z* 131 which indicated that 15 Da were lost directly from the parent deprotonated molecule as the methyl group. Losses of 28 Da as CO from hydroxy-aromatic compounds are typical when conducting ESI negative ionization mass analyses (Xu et al. 2004). Direct loss of 44 Da occurred from the parent deprotonated molecule-represented by fragment *m/z* 102-and this event provided strong evidence that the location of 3-methylindole molecule oxidation had occurred at the 2-carbon position (Fig. 3a). This occurred through ionization and collapse of the pyrrole ring through loss of $CONH_2$. This type of fragmentation has been reported previously during ESI fragmentation of indole oxidized at carbon position 2 (Fukuoka et al. 2015). Based upon these results, a molar mass of 147 Da and a molecular formula of $C_9H_9NO$, product I was assigned an identity of 3-methyl-1*H*-indol-2-ol. As shown in Fig. 3b, c, results of product ion analyses of products II and III revealed mass spectra that were similar to each other, indicating losses of 15 Da (*m/z* 131) as the methyl group and 28 Da (*m/z* 118) as CO from each from the parent deprotonated molecules. These results provided evidence for two more monohydroxylated products of 3-methylindole where

oxidation had occurred on the 6-membered aromatic ring. Details of these and proceeding product ion scan analyses are given in Table 1. Two deprotonated molecules were revealed that corresponded to biotransformation products IV and V, $[M-H]^- = 162$ each, that eluted at 6.0 and 6.6 min respectively. As shown in Fig. 4a, b, results of product ion scan analyses of these biotransformation products showed loss of water (*m/z* 144), loss of CO (*m/z* 134), and loss of water plus CO (or indole anion; *m/z* 116) from the deprotonated molecule, in addition to aniline anion (*m/z* 92). Taken together these data provided evidence for a 3-methylindole molecule that was oxidized in two positions. For both biotransformation products, losses of 15 Da each (*m/z* 147) were also documented from the parent deprotonated molecules and these results indicated that the location of the oxidation events on the 3-methylindole molecule had not occurred at the methyl group. Analyses further revealed a strong diagnostic fragment at *m/z* 118 for biotransformation product V that was of similar strength to *m/z* 116 (Fig. 4b). It resulted from a loss of 44 Da from the parent deprotonated molecule and provided strong evidence for the position of one of the two hydroxyl groups on this biotransformation product to be located at carbon position 2 on the *N*-heterocyclic ring as shown in Fig. 4b. The second hydroxyl group was located on the carbocyclic ring in one of carbon positions 4 through 7. In the case of product IV, both oxidations occurred on the carbocyclic ring of 3-methylindole as shown in Fig. 4a. Overall, consideration of these results, molar masses of 163 Da each and molecular formulae of $C_9H_9NO_2$, these two biotransformation products represented dihydroxylated 3-methylindole compounds.

Shown in Fig. 4c is the mass spectrum of the deprotonated molecule that corresponded to product VI, $[M-H]^- = 160$, $t_R = 6.3$ min which revealed a loss of 44 Da as $CO_2$ from the parent deprotonated molecule and production of the indole anion (*m/z* 116). Based upon these results, a molar mass of 161 Da and a molecular formula of $C_9H_7NO_2$, product VI was assigned to be 1*H*-indole-3-carboxylic acid and this is in agreement with previous work in regard to the mass fragmentation of 1*H*-indole-3-carboxylic acid whereby *m/z* 116 was a major product ion (Powers 1968). Detection of 1*H*-indole-3-carboxylic acid in strain KK10 extracts provided evidence for another pathway of 3-methylindole biodegradation by this strain through oxidation of the methyl group of 3-methylindole; most likely through production of indole-3-carbinol. The pathways of biotransformation of 3-methylindole by strain KK10 through both the *N*-heteroaromatic and carbocyclic rings is summarized as part of Fig. 5 and includes results discussed in the proceeding sections.

Finally, product ion scan analyses of the deprotonated molecule $[M-H]^- = 144$, also yielded indole anion at *m/z* 116 as the sole fragmentation product through a loss of 28 Da as CO (Table 1). By consideration of a molar mass of 145 Da and a molecular formula of $C_9H_7NO$, product VII was assigned to be 1*H*-indole-3-carbaldehyde which eluted at 6.6 min. The detection of 1*H*-indole-3-carbaldehyde provided further support for the indole-3-carbinol pathway by strain KK10 as shown in Fig. 5.

## Aromatic ring fission of 3-methylindole occurred through the carbocyclic ring

Biotransformation product VIII eluted at $t_R = 5.7$ - min and corresponded to the deprotonated molecule, $[M-H]^- = 194$. Figure 6a shows the proposed molecular structure and supporting fragmentation pattern obtained from CID analyses whereby losses of 44 Da (*m/z* 150), 70 Da (*m/z* 124) and 88 Da (*m/z* 106) each occurred from the parent deprotonated molecule. Respectively these fragmentation ions indicated losses of $CO_2$, $CO_2$ plus an ethyne group ($C_2H_2$), and $2CO_2$ from biotransformation product VIII. Not only did this pattern of fragmentation provide strong support that this was an *ortho*-cleavage product of carbocyclic aromatic ring fission of 3-methylindole (Fig. 6a), but through detection of the loss of the ethyne moiety, it indicated that product VIII was formed from either 3-methyl-1*H*-indole-4,5- or -6,7-diol. Considering the fragmentation pattern, a molar mass of 195 Da and a molecular formula of $C_9H_9NO_4$, this compound was assigned an identity of 2- or 3-[(*E* or *Z*)-2-carboxyvinyl]-4-methyl-1*H*-pyrrole-3- or -2-carboxylic acid (Table 1). This biotransformation product occurred through initial oxidative attack by strain KK10 at the 4,5- or 6,7-carbon positions of 3-methylindole to produce 3-methyl-4,5-dihydro-1*H*-indole-4,5-diol and/or 3-methyl-6,7-dihydro-1*H*-indole-6,7-diol followed by 3-methyl-1*H*-indole-4,5- and/or -6,7-diol as described in Fig. 5 whereby structures for oxidation through 4,5 oxidation are given.

**Table 1** Results of product ion scan analyses of biotransformation products related to 3-methylindole biodegradation

| Parent deprotonated molecule[a] $[M-H]^-$ | Product number | $t_R^b$ (min) | CID[c] (eV) | Diagnostic fragments from product ion scan analyses (% relative intensity) | Identity assignment |
|---|---|---|---|---|---|
| 146 | I | 3.9 | 8 | 146 ($M^-$, 100), 131 ($M^-$–$CH_3$, 5), 128 ($M^-$–$H_2O$, 23), 118 ($M^-$–CO, 30), 102 ($M^-$–$CONH_2$, 8), 87 ($M^-$–$CH_3$–$CONH_2$, 7) | 3-methyl-1*H*-indol-2-ol |
| 146 | II | 5.8 | 8 | 146 ($M^-$, 100), 131 ($M^-$–$CH_3$, 19), 118 ($M^-$–CO, 10) | Carbocyclic ring monohydroxylation product of 3-methylindole |
| 146 | III | 6.3 | 8 | 146 ($M^-$, 100), 131 ($M^-$–$CH_3$, 44), 118 ($M^-$–CO, 8) | Carbocyclic ring monohydroxylation product of 3-methylindole |
| 162 | IV | 6.0 | 30 | 162 ($M^-$, 7), 147 ($M^-$–$CH_3$, 16), 144 ($M^-$–$H_2O$, 47), 134 ($M^-$–CO, 26), 116 ($M^-$CO–$H_2O$, 16), 92 ($C_6H_6N^-$, 100) | 3-methyl-1*H*-indole-4,5- and/or -6,7-diol |
| 162 | V | 6.6 | 30 | 162 ($M^-$, 8), 147 ($M^-$–$CH_3$, 27), 144 ($M^-$–$H_2O$, 56), 134 ($M^-$–CO, 34), 118 ($M^-$–$CONH_2$, 17), 116 ($M^-$CO–$H_2O$, 21), 92 ($C_6H_6N^-$, 100) | 3-methyl-1*H*-indole-2,(4-,5-,6-, and/or 7-)diol |
| 160 | VI | 6.3 | 8 | 160 ($M^-$, 100), 116 ($M^-$–$CO_2$, 55) | 1*H*-indole-3-carboxylic acid |
| 144 | VII | 6.6 | 15 | 144 ($M^-$, 100), 116 ($M^-$–CO, 17) | 1*H*-indole-3-carbaldehyde |
| 194 | VIII | 5.7 | 8 | 194 ($M^-$, 38), 163 (4), 150 ($M^-$–$H_2O$, 4), 152 (9), 124 ($M^-$–$CO_2$–$C_2H_2$, 7), 106 ($M^-$–$2CO_2$, 12) | 2- or 3-[(*E*)-2-carboxyvinyl]-4-methyl-1*H*-pyrrole-3- or -2-carboxylic acid |
| 168 | IX | 3.1 | 8 | 168 ($M^-$, 100), 153 ($M^-$–$CH_3$, 3), 150 ($M^-$–$H_2O$, 3), 136 (5), 124 ($M^-$–$CO_2$, 45), 80 ($M^-$–$CO_2$, 19) | 4-methyl-1*H*-pyrrole-2,3-dicarboxylic acid |
| 198 | X | 4.2 | 8 | 198 ($M^-$, 58), 154 ($M^-$–$CO_2$, 100), 110 ($M^-$–$2CO_2$, 57) | 1*H*-pyrrole-2,3,4-tricarboxylic acid |
| 112 | XI | 2.7 | 8 | 112 ($M^-$, 100), 97 ($M^-$–$CH_3$, 81), 94 ($M^-$–$H_2O$, 25), 80 ($C_5H_6N^-$, 29) | 4-methyl-1*H*-pyrrole-2,3-diol |
| 307 | XII | 6.9 | 8 | 307 ($M^-$, 100), 160 ($C_9H_6NO_2^-$, 25), 146 ($C_9H_8NO^-$, 14) | Alkylated diindole dimer, monohydroxy and carboxyl moieties |
| 309 | XIII | 7.8 | 20 | 309 ($M^-$, 100), 249 (26), 209 (44), 162 ($C_9H_8NO_2^-$, 81), 146 ($C_9H_8NO^-$,73), 134 ($C_9H_8NO_2^-$–CO, 79) | Alkylated diindole dimer, mono-plus di-hydroxylated moieties |
| 293 | XIV | 8.9 | 8 | 293 ($M^-$, 100), 275 ($M^-$–$H_2O$, 7), 251 (8), 247 ($M^-$–$H_2O$–CO, 9), 231 (4), 162 ($C_9H_8NO_2^-$, 4), 146 ($C_9H_8NO^-$, 9), 130 ($C_9H_8N^-$, 4) | Alkylated diindole dimers of un-, mono- and di-hydroxylated moieties |

[a] Parent deprotonated molecules are listed in the order that they are described in the text

[b] $t_R$, retention time

[c] Collision induced dissociation energy

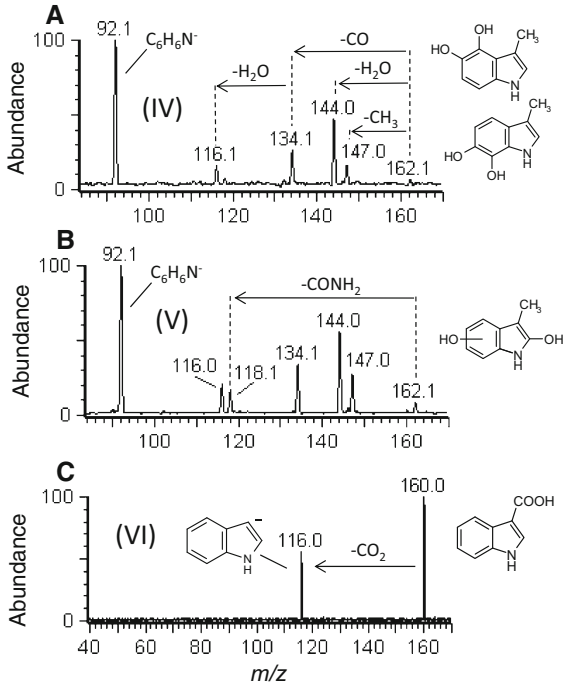


**Fig. 4** ESI(−)–MS/MS spectra acquired by product ion scan analyses of three oxidized 3-methylindole biotransformation products. **a** Product IV, $t_R = 6.0$ min, corresponding to $[M-H]^- = 162$; **b** Product V, $t_R = 6.6$ min, corresponding to $[M-H]^- = 162$; **c** Product VI, $t_R = 6.3$ min, corresponding to $[M-H]^- = 160$. The chemical structures proposed for these biotransformation products are also shown

Shown in Fig. 6b is the mass spectrum that resulted from CID analysis of product IX, $[M-H]^- = 168$, which eluted at 3.1 min. Two abundant fragmentation ions at *m/z* 124 and *m/z* 80 were revealed and this fragmentation pattern indicated double losses of $CO_2$ from the parent deprotonated molecule which provided evidence for another *ortho*-type cleavage product of 3-methylindole. Losses of 15 Da and 18 Da as $CH_3$ and $H_2O$ respectively from the parent deprotonated molecule were also detected (Table 1). Based upon these data, a molar mass of 169 Da, and a molecular formula of $C_7H_7NO_4$, product IX was proposed to be a dicarboxylic acid of 1*H*-pyrrole, 4-methyl-1*H*-pyrrole-2,3-dicarboxylic acid. Biotransformation product X, $[M-H]^- = 198$, $t_R = 4.2$ min was weakly detected, however product ion scan analysis revealed a mass spectrum with clear product ions at *m/z* 154 and *m/z* 110 which again indicated double losses of $CO_2$ from the parent deprotonated molecule (Fig. 6c). This fragmentation pattern matched identically to the fragmentation pattern reported by Glass et al. (2012) during negative ionization mass spectrometry analysis of 1*H*-pyrrole-2,3,4-tricarboxylic acid. Considering a molar mass of 199 Da and molecular formula of $C_7H_5NO_6$, it was concluded that product X was 1*H*-pyrrole-2,3,4-tricarboxylic acid and this compound represented a third downstream *ortho*-cleavage product of carbocyclic aromatic ring biotransformation of 3-methylindole. Additionally, detection of this product provided more evidence for an indole-3-carbinol pathway by this strain. Product X was formed through either initial oxidation events on the carbocyclic ring or through the methyl group of 3-methylindole as summarized in Fig. 5. The detection of biotransformation products VIII, IX and X by LC/ESI–MS/MS provided direct evidence of 3-methylindole biotransformation via ring cleavage by this bacterium. In this case, results supported a mechanism of lateral dioxygenation followed by *ortho*-ring fission. Oxidation through carbon positions 4 and 5 of 3-methylindole was analogous to 1,2-carbon position dioxygenation by bacteria of the structurally analogous *N*-heterocyclic aromatic chemical, 9*H*-carbazole (Seo et al. 2006). 9*H*-carbazole is comprised of indole fused at the 2–3 positions to a 6-membered aromatic ring and this 6-membered ring is biotransformed through lateral dioxygenation in an identical manner as shown for 3-methylindole by strain KK10. The mechanism involved ring cleavage to produce the biotransformation products 4-(3-hydroxy-1*H*-indol-2-yl)-2-oxobut-3-enoic acid and 2-(2-carboxy-vinyl)-1*H*-indole-3-carboxylic acid (Seo et al. 2006). The latter product of *ortho*-ring fission, 2-(2-carboxy-vinyl)-1*H*-indole-3-carboxylic acid, was the structural analogue to biotransformation product VIII from 3-methylindole biodegradation by strain KK10. Detection of bacterial biotransformation products that indicated lateral dioxygenation and *ortho*-ring fission of the structurally-related aromatic heterocycles: indole, dibenzofuran and dibenzothiophene have recently been reported for *Cupriavidus*, *Pseudomonas*, and *Arthrobacter* spp. respectively (Seo et al. 2006; Li et al. 2009; Fukuoka et al. 2015).

Finally, a product with a low molar mass of 113 Da was weakly detected at 2.7 min, product XI, and results of CID analysis combined with consideration of a molecular formula of $C_5H_7NO_2$ supported that it was 4-methyl-1*H*-pyrrole-2,3-diol, a potential downstream product of 4-methyl-1*H*-pyrrole-2,3-dicarboxylic acid

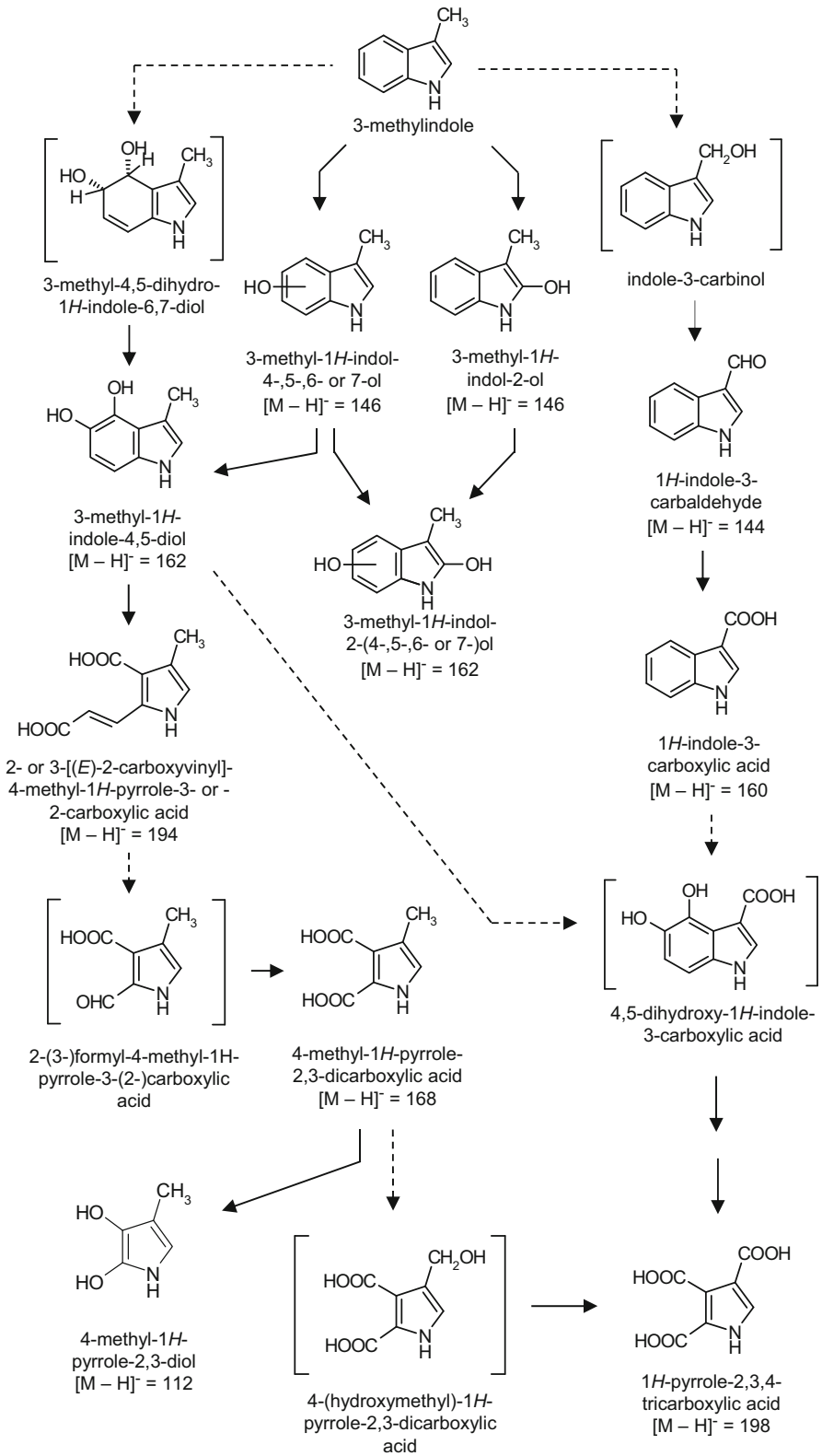


**Fig. 5** Pathways proposed for the biotransformation of 3-methylindole by *Cupriavidus* sp. strain KK10. Products in *brackets* are hypothesized intermediates

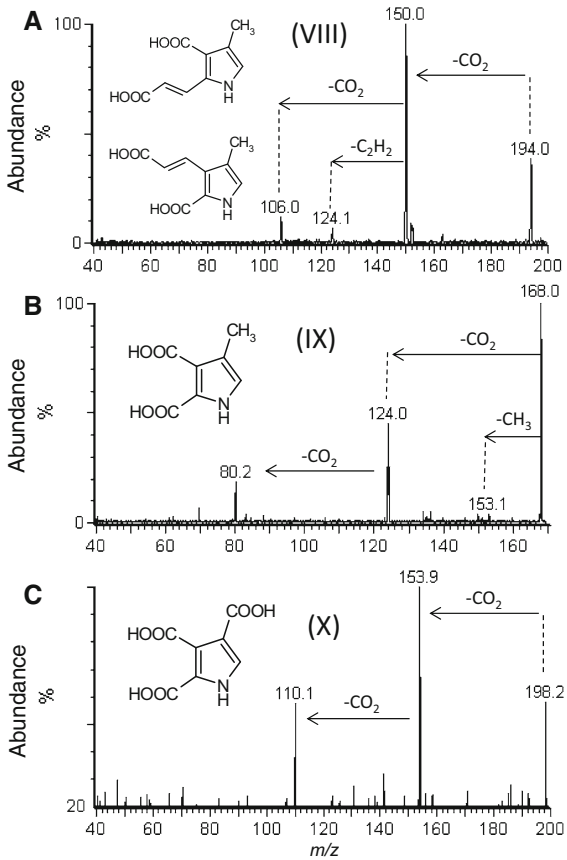


**Fig. 6** ESI(−)–MS/MS spectra acquired by product ion scan analyses of ring cleavage products of 3-methylindole biotransformation. **a** Product VIII, $t_R = 5.7$ min, corresponding to $[M-H]^- = 194$; **b** Product IX, $t_R = 3.1$ min, corresponding to $[M-H]^- = 168$; **c** Product X, $t_R = 4.2$ min, corresponding to $[M-H]^- = 198$. Note the baseline of (**c**) is raised 20 %. The chemical structures proposed for these biotransformation products are also shown

(Table 1, Supplementary Fig. S2, Fig. 5). Detection of this diol pointed towards potential pyrrole ring opening even though direct detection of ring-fission products of pyrrolic compounds was not possible. To test whether strain KK10 was able to catalyze ring fission however, the commercially available chemical standard, 1*H*-pyrrole-2-carboxylic acid was investigated in a biodegradation assay. It was rapidly depleted from culture media within 24 h and the deprotonated molecule $[M-H]^- = 129$ was detected in culture extracts. Results of CID analyses for this product were consistent with its identity as the pyrrole ring fission product, 2-oxoglutarate semialdehyde (Supplementary Fig. S3). From these results that indicated a lack of accumulation of low molar mass products during 3-methylindole biodegradation combined with the detection of 4-methyl-1*H*-pyrrole-2,3-diol, it was concluded that cometabolic fission of the 5-membered ring had occurred. At the same time, direct evidence of pyrrole ring fission by examination of a structurally similar compound added support to this hypothesis.

## Indole biotransformation pathway convergence through decarboxylation of 1H-indole-3-carboxylic acid did not occur

Oxidation of the methyl group of 3-methylindole was shown to occur through 1*H*-indole-3-carboxylic acid. If decarboxylation of this product to produce mono-oxidized forms of indole resulted, production of indole pathway biotransformation products such as isatin and salicylic acid would be expected to have occurred (Eaton and Chapman 1995; Fukuoka et al. 2015). At the same time, if downstream biotransformation were not possible, at least the formation of indigoids such as indirubin and indigo would occur through dimerization of oxidized indoles (Eaton and Chapman 1995). Considering this, analyses of authentic standards of isatin, salicyclic acid, indirubin and indigo were conducted to determine their retention times and mass fragmentation patterns with the purpose to search for these products in 3-methylindole biodegradation extracts (Supplementary Table S1). For example, the value for the deprotonated molecule of isatin, $[M-H]^- = 146$, was identical to some products of 3-methylindole. Results of LC/ESI(-)-MS/MS analyses showed however that mass spectra and retention time data were different and indicated that these compounds were not detected in culture fluid extracts of 3-methylindole biodegradation by strain KK10. It was concluded that indole-type biotransformation pathways through the *N*-heterocyclic aromatic ring of 3-methylindole were not utilized during its biotransformation.

## Identification of high molar mass products confirms upper pathways

Precursor ion scan analyses of $[M-H]^- = 146$ revealed relatively high molar mass biotransformation products that eluted latest compared to other products and corresponded to the deprotonated molecules $[M-H]^- = 307$, 309, and 293, products XII, XIII and XIV, $t_R = 6.9$, 7.8 and 8.9 min respectively

(Table 1). Results of product ion scan analysis of product XII, $[M-H]^- = 307$, revealed two fragments at *m/z* 160 and *m/z* 146 which appeared to be oxidized 3-methylindole anions (Supplementary Fig. S4A) that occurred through fragmentation of biotransformation products that were formed through dimerization of products VI, 1*H*-indole-3-carboxylic acid and monohydroxylation products of 3-methylindole (e.g., products II and III). These results added further support to the direct detection of 1*H*-indole-3-carboxylic acid and the monohydroxylation products of 3-methylindole in culture media as described above. Considering a molecular formula of $C_{18}H_{16}N_2O_3$ and a molar mass of 308 Da, it was concluded that this was a dimer formed through carbon position 2 as shown in Supplementary Fig. S4A. It is known that indoles such as 3-methylindole that are substituted at the 3-carbon position may dimerize through electrophilic substitution at the 2-carbon position (Nolan and Hammer 1960; Smith and Walters 1961). Results of product ion scan analyses of biotransformation products XIII and XIV, that corresponded to $[M-H]^- = 309$ and $[M-H]^- = 293$, indicated that these were also dimers. Analysis of the peak that corresponded to product XIII yielded diagnostic fragmentation ions at *m/z* 162, *m/z* 146, and *m/z* 134 and indicated that it represented dimers that consisted of both a single- and double-oxidized 3-methylindole molecule through carbon position 2 as described in Table 1. A representative structure is shown in Supplementary Fig. S4B. In the case of product XIV, main diagnostic fragmentation ions were revealed at *m/z* 162, *m/z* 146, and *m/z* 130 and corresponded to anions of 3-methylindole, and hydroxylated and dihydroxylated 3-methylindoles respectively (Supplementary Fig. S4C; Table 1). Taken together, results of fragmentation analyses of these high molar mass dimerization products provided confirmation for the production of mono- and dioxidized 3-methylindoles that were proposed for the upper pathways of 3-methylindole biotransformation by strain KK10.

## Discussion

There is interest to understand the environmental fate of 3-methylindole due to its potentially deleterious effects on human health and quality of life, in combination with its mobility and potential to persist (Yager et al. 2014). Its reported persistence may be due in part to the location of the methyl substituent on the *N*-heterocyclic ring, which hinders biotransformation of the molecule through a 2,3-carbon position oxidation pathway and ring fission as has been determined to occur during indole biotransformation (Fujioka and Wada 1968). Overall, the aerobic biodegradation pathways of 3-methylindole by bacteria are unknown and this appears to be due in part to the toxicity of this compound towards microorganisms (Yokoyama and Carlson 1979; Deslandes et al. 2001; Regal et al. 2001; Choi et al. 2013). In the investigation herein, results of experiments showed that when growing on glycerol as the sole source of carbon and energy, *Cupriavidus* sp. strain KK10 biodegraded a high concentration of 3-methylindole in 24 h. Interestingly, growth experiments showed that strain KK10 could not utilize 3-methylindole as a carbon source. Cometabolism of PAHs by pure strains of bacteria is known to occur mostly when cells are grown on structurally similar substrates (Kanaly and Harayama 2000), however we are unaware of studies where glycerol stimulated a bacterial isolate to biodegrade PAHs in this capacity. Currently, unwanted large quantities of glycerol are produced throughout the world as the main by-product of biodiesel production and this situation is posing disposal challenges to the industry (Quispe et al. 2013). One proposed method of glycerol disposal involves its utilization as a feedstock for microorganisms to create blends with other waste streams to mitigate multiple challenges (Samul et al. 2014). Results described in this work have established that strain KK10 may be useful in this capacity.

Results of quantitative 3-methylindole biodegradation analyses were confirmed by the elucidation of aerobic biotransformation pathways for the first time. At least fourteen products, including aromatic ring-fission products, were identified by LC/ESI(−)–MS/MS analyses thusly demonstrating the complexity of 3-methylindole biotransformation. Results showed that oxidation of 3-methylindole by strain KK10 occurred on multiple locations on the 3-methylindole molecule, i.e., on the 6-membered ring, on the 2-carbon position of the *N*-heterocyclic ring, and on the 3-carbon position methyl group. Three of these biotransformation products, II, III and VI, occurred in high relative abundances after 6 h and then rapidly

decreased from culture media which confirmed the transience of these products during transformation (Fig. 2). By 24 h, biotransformation product IV occurred in the greatest abundance and indicated that dihydroxylation of the 6-membered aromatic ring was occurring to a large extent. By 48 h, it was not detected and this indicated that it was rapidly transformed to downstream products.

Identification in culture media of 1*H*-indole-3-carboxylic acid, [M−H$^-$ = 160], and 1*H*-indole-3-carbaldehyde, [M−H$^-$ = 144], provided direct evidence for oxidative attack at the methyl group. 1*H*-Indole-3-carbaldehyde biotransformation product VII coeluted with product V, and the amounts of both of these products were reduced by 24 h and not detected after 48 h (Fig. 2). Production of product V most likely occurred through indole-3-carbinol—which was not detected (Fig. 5). At the same time, although 1*H*-indole-3-carboxylic acid was detected (product VI), isatin and indigoids were not. These results indicated that decarboxylation of 1*H*-indole-3-carboxylic acid en route to the production of 3-indoxyl/3-oxindole or 2,3-dihydroxy-indole had not occurred. Indigo may be formed through hydroxylation of indole at carbon position 3 for example (Mermod et al. 1986). The potential downstream product, salicylic acid, was also not detected. This result was in agreement with growth results that showed that strain KK10 was unable to use 3-methylindole as a source of carbon for growth. Taken together, it was concluded that the 3-methylindole biotransformation pathways did not converge with known indole biotransformation pathways (Eaton and Chapman 1995; Fukuoka et al. 2015).

Until now, the only aerobic biotransformation pathway published for 3-methylindole biodegradation by a bacterium, *Pseudomonas aeruginosa* Gs, involved oxidation via the methyl group whereby it was proposed to proceed through anthranilic acid even though it was not detected (Yin and Gu 2006). Two metabolites, indoline-3-carboxylic acid and indoline-3-ol were proposed based upon the detection of only deprotonated molecules in culture extracts. Other metabolites may have been present, however the authors reported that their methods were limited by sensitivity. Biotransformation through anthranilic acid by strain Gs was indicative of convergence of the 3-methylindole pathway with the known indole biotransformation pathways (Fujioka and Wada 1968). As explained previously, 3-methylindole biotransformation by strain KK10 did not converge with the indole pathways. Recently, biodegradation of 3-methylindole by another Proteobacterium, *Rhodopseudomonas palustris* was reported (Sharma et al. 2014). *R. palustris* was isolated from a swine waste lagoon and biodegraded near 50 % of 0.1 mg/L 3-methylindole in 72 h, however metabolites were not identified.

In regard to direct oxidation of the aromatic ring(s) of 3-methylindole, oxidative attack at the 2- and 3-carbon atoms by an aerobic soil bacterium to produce 2-oxo-3-methyl-3-hydroxyindoline was proposed by Fujioka and Wada (1968). Under anaerobic conditions, 3-methylindole biotransformation by methanogenic and sulfate-reducing consortia resulted in the production of 3-methyloxindole, which also occurs through oxidation of the 2-carbon position of 3-methylindole. It was the only metabolite reported in all cases however (Gu and Berry 1991, 1992; Gu et al. 2002). Ring-opening by consortia was proposed to occur through production of α-methyl-2-aminobenzene acetic acid en route to mineralization however it was not detected (Gu et al. 2002). Contrastively, evidence for oxidative attack on the aromatic rings of 3-methylindole by strain KK10 was provided for by the direct detection of monooxidized forms of 3-methylindole: 2-hydroxy-3-methylindole via the 5-membered ring, and singly-hydroxylated 3-methylindoles via the 6-membered ring. At the same time, direct detection of dioxidized biotransformation products such as product V, (Fig. 4), showed that two separate monooxygenation events occurred on the 3-methylindole molecule on both aromatic rings. Indeed, based upon the results of previous studies (Fujioka and Wada 1968; Gu and Berry 1991, 1992; Gu et al. 2002) and this study, the carbon located at the 2-carbon position of 3-methylindole appears amenable to oxidation by bacterial enzymes. In the case of strain KK10 however, another pathway for 3-methylindole biotransformation through a 3-methylindole-dihydrodiol was proposed based upon detection of product(s) IV (Fig. 4) and detection of subsequent downstream ring-opened biotransformation products (Fig. 5).

Previous studies have only reported oxidation of 3-methylindole through the 5-membered ring, and even so, ring cleavage products have not yet been shown. In this study, identification of product VIII, the ring cleavage product 2-(3-)[carboxyvinyl]-4-methyl-1*H*-pyrrole-3-(2-) carboxylic acid, [M−H$^-$ = 194], (Fig. 6) provided strong support for a 4,5- or 6,7- oxidative pathway of

3-methylindole biotransformation. It also showed that *ortho*-ring cleavage of the carbocyclic ring of 3-methylindole had occurred by strain KK10. These results supported previous work with this strain that indicated that dioxygenation and ring cleavage of the carbocyclic aromatic ring of indole had occurred (Fukuoka et al. 2015). Furthermore, two downstream products of intradiol ring cleavage, 4-methyl-1*H*-pyrrole-2,3-dicarboxylic acid and 1*H*-pyrrole-2,3,4-tricarboxylic acid were also identified in culture media and their detection provided more support for a mechanism of biodegradation through 3-methyl-4,5-(and/or -6,7-)dihydroxy-1*H*-indole as shown in Fig. 5. Pyrrolic compounds may be considered to be toxic and in this investigation, a small relative increase in product IX, 4-methyl-1*H*-pyrrole-2,3-dicarboxylic acid may have occurred (Fig. 2c). However, large accumulation of these dead-end products was not observed which indicated that they occurred transiently during biotransformation. Although pyrrole ring-fission products were not directly detected during 3-methylindole biodegradation, a potential downstream product of 4-methyl-1*H*-pyrrole-2,3-dicarboxylic acid was weakly detected and identified as 4-methyl-1*H*-pyrrole-2,3-diol—which may be expected to be rapidly transformed to ring-opened products. In separate experiments, strain KK10 was found to rapidly biotransform the pyrrole carboxylic acid, 1*H*-pyrrole-2-carboxylic acid, and the ring fission product, 2-oxoglutarate semialdehyde was detected. Taken together, the results of these experiments, combined with the observation that large accumulation of pyrrolic products did not occur in culture media, indicated that strain KK10 was most likely capable of pyrrole ring fission during 3-methylindole biodegradation. Further investigation shall be necessary to add to the results in this study. Interestingly, ring cleavage of 6-membered ring PAHs by *ortho*-type mechanisms are still not much reported and there is little known about the enzymes involved. The proposed product(s) of carbocyclic intradiol ring cleavage of 3-methylindole, 2-(3-)[carboxyvinyl]-4-methyl-1*H*-pyrrole-3-(2-)carboxylic acid(s), that contained an aromatic carboxyvinyl substituent, are analogous to PAH *ortho*-ring fission products detected from *Mycobacterium* and *Sphingobium* spp. (Moody et al. 2001; Kunihiro et al. 2013; Maeda et al. 2014). However, compared to the known biodegradative capabilities of members from these genera towards hazardous polyaromatic pollutants, members of the genus *Cupriavidus* have not been studied.

Identification of high molar mass products that were formed through dimerization reactions of 3-methylindole and 3-methylindole biotransformation products provided further evidence to support the structures of products proposed in the upper pathways, i.e., the pre-ring fission pathways. Identification of dimers showed that the production of mono- and dihydroxylated 3-methylindoles and 1*H*-indole-3-carboxylic acid in the culture medium had occurred and taken together with direct identification of biotransformation products described above, these results allowed for the construction of upper and lower pathways for biotransformation through the *N*-heterocyclic and carbocyclic rings of 3-methylindole (Fig. 5). Biotransformation products that corresponded to relatively high molar mass compounds also likely contributed to removal of some products through polymerization reactions (data not shown). At the same time, that indigoid compounds were not detected provided another line of evidence that biotransformation had not occurred through production of oxindole compounds.

## Conclusions

This work extended our understanding of 3-methylindole biodegradation by bacteria; specifically for a member of the genus *Cupriavidus*. Biotransformation pathways for 3-methylindole were constructed that included evidence for oxidation of both aromatic rings, including ring cleavage. Further understanding in regard to the manner by which glycerol stimulates biodegradation is warranted. Worldwide, biodiesel production results in large amounts of glycerol by-product and there is interest in microorganisms that utilize glycerol as a source of carbon and energy and which at the same time may facilitate biodegradation of pollutants in other waste streams. This research has showed that strain KK10 is a promising candidate for further evaluation in this regard.

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## References

Atlas RM (1993) Handbook of microbiological media. CRC Press, Boca Raton

Beier RC, Anderson RC, Krueger NA, Edrington TS, Callaway TR, Nisbet DJ (2009) Effect of nitroethane and

nitroethanol on the production of indole and 3-methylindole (skatole) from bacteria in swine feces by gas chromatography. J Environ Sci Health Part B 44:613–620

Botalova O, Schwarzbauer J (2011) Geochemical characterization of organic pollutants in effluents discharged from various industrial sources to riverine systems. Water Air Soil Pollut 221:77–98

Choi SH, Kim Y, Oh S, Oh S, Chun T, Kim SH (2013) Inhibitory effect of skatole (3-methylindole) on enterohemorrhagic *Escherichia coli* O157:H7 ATCC 43894 biofilm formation mediated by elevated endogenous oxidative stress. Lett Appl Microbiol 58:454–461

Cook KL, Rothrock MJ, Lovanh N, Sorrell JK, Loughrin JH (2010) Spatial and temporal changes in the microbial community in an anaerobic swine waste treatment lagoon. Anaerobe 16:74–82

De la Torre AI, Jiménez JA, Carballo M, Fernandez C, Roset J, Muñoz MJ (2000) Ecotoxicological evaluation of pig slurry. Chemosphere 41:1629–1635

Deslandes B, Gariépy C, Houde A (2001) Review of microbiological and biochemical effects of skatole on animal production. Livest Prod Sci 71:193–200

Diamond JM, Latimer HA, Munkittrick KR, Thorton KW, Bartell SM, Kidd KA (2011) Prioritizing contaminants of emerging concern for ecological screening assessments. Environ Toxicol Chem 30:2385–2394

Ducey TF, Hunt PH (2013) Microbial community analysis of swine wastewater anaerobic lagoons by next-generation DNA sequencing. Anaerobe 21:50–57

Eaton RW, Chapman PJ (1995) Formation of indigo and related compounds from indolecarboxylic acids by aromatic acid-degrading bacteria: chromogenic reactions for cloning genes encoding dioxygenases that act on aromatic acids. J Bacteriol 177:6983–6988

Fujioka M, Wada H (1968) The bacterial oxidation of indole. Biochim Biophys Acta 158:70–78

Fukuoka K, Tanaka K, Ozeki Y, Kanaly RA (2015) Biotransformation of indole by *Cupriavidus* sp. strain KK10 proceeds through *N*-heterocyclic- and carbocyclic-aromatic ring cleavage and production of indigoids. Int Biodeterior Biodegrad 97:13–24

Glass K, Ito S, Wilby PR, Sota T, Nakamura A, Bowers CR, Vinther J, Dutta S, Summons R, Briggs DEG, Wakamatsu K, Simon JD (2012) Direct chemical evidence for eumelanin pigment from the Jurassic period. Proc Natl Acad Sci USA 109:10218–10223

Gu J-D, Berry DF (1991) Degradation of substituted indoles by an indole-degrading methanogenic consortium. Appl Environ Microbiol 57:2622–2627

Gu J-D, Berry DF (1992) Metabolism of 3-methylindole by a methanogenic consortium. Appl Environ Microbiol 58:2667–2669

Gu J-D, Fan Y, Shi H (2002) Relationship between structures of substituted indolic compounds and their degradation by marine anaerobic microorganisms. Mar Pollut Bull 45:379–384

Kanaly RA, Harayama S (2000) Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059–2067

Kanaly RA, Watanabe K (2004) Multiple mechanisms contribute to the biodegradation of benzo[a]pyrene by petroleum-derived multicomponent nonaqueous-phase liquids. Environ Toxicol Chem 23:850–856

Kanaly RA, Bartha R, Fogel S, Findlay M (1997) Biodegradation of [$^{14}$C]benzo[*a*]pyrene added in crude oil to uncontaminated soil. Appl Environ Microbiol 63:4511–4515

Kanaly RA, Bartha R, Watanabe K, Harayama S (2000) Rapid mineralization of benzo[*a*]pyrene by a microbial consortium growing on diesel fuel. Appl Environ Microbiol 66:4205–4211

Kunihiro M, Ozeki Y, Nogi Y, Hamamura N, Kanaly RA (2013) Benz[*a*]anthracene biotransformation and production of ring fission products by *Sphingobium* sp. strain KK22. Appl Environ Microbiol 79:4410–4420

Li G, Wang X, Yin G, Gai Z, Tang H, Ma C, Deng Z, Xu P (2009) New metabolites of dibenzofuran cometabolic degradation by a biphenyl-cultivated Pseudomonas putida strain B6-2. Environ Sci Technol 43:8635–8642

Maeda AH, Nishi S, Hatada Y, Ozeki Y, Kanaly RA (2014) Biotransformation of the high-molecular-weight polycyclic aromatic hydrocarbon (PAH) benzo[*k*]fluoranthene by *Sphingobium* sp. strain KK22 and identification of new products of non-alternant PAH biodegradation by liquid chromatography electrospray ionization tandem mass spectrometry. Microbial Biotechnol 7:114–129

Mermod N, Harayama S, Timmis KN (1986) New route to bacterial production of indigo. Nat Biotechnol 4:321–324

Moody JD, Freeman JP, Doerge DR, Cerniglia CE (2001) Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. strain PYR-1. Appl Environ Microbiol 67:1476–1483

Nolan WE, Hammer CF (1960) Mixed indole dimers, trimers, and their acyl derivatives. J Org Chem 25:1525–1535

Powers JC (1968) Mass spectrometry of simple indoles. J Org Chem 33:2044–2050

Quispe CAG, Coronado CJR, Carvalho JA (2013) Glycerol: production, consumption, prices, characterization and new trends in combustion. Renew Sustain Energy Rev 27:475–493

Regal KA, Laws GM, Yuan C, Yost GS, Skiles GL (2001) Detection and characterization of DNA adducts of 3-methylindole. Chem Res Toxicol 14:1014–1024

Samul D, Leja K, Grajek W (2014) Impurities of crude glycerol and their effect on metabolite production. Ann Microbiol 64:891–898

Schüssler W, Nitschke L (1999) Death of fish due to surface water pollution by liquid manure or untreated wastewater: analytical preservation of evidence by HPLC. Water Res 33:2884–2887

Seo J-S, Keum Y-S, Cho IK, Li QX (2006) Degradation of dibenzothiophene and carbazole by Arthrobacter sp. P1-1. Int Biodeterior Biodegrad 58:36–43

Sharma N, Doerner KC, Alok PC, Choudhary M (2014) Skatole remediation potential of *Rhodopseudomonas palustris* WKU-KDNS3 isolated from an animal waste lagoon. Lett Appl Microbiol 60:298–306

Smith GF, Walters AE (1961) Indoles. Part V: 3-alkylindole dimers. J Chem Soc 940–943

Trabue S, Scoggin K, McConnell L, Maghirang R, Razote E, Hatfield J (2011) Identifying and tracking key odorants from cattle feedlots. Atmos Environ 45:4243–4251

Wu JJ, Park S, Hengemuehle SM, Yokoyama MT, Person HL, Gerrish JB, Masten SJ (1999) The use of ozone to reduce

the concentration of malodorous metabolites in swine manure slurry. J Agr Eng Res 72:317–327

Xu X, Zhang J, Zhang L, Liu W, Weisel CP (2004) Selective detection of monohydroxy metabolites of polycyclic aromatic hydrocarbons in urine using liquid chromatography/triple quadrupole tandem mass spectrometry. Rapid Commun Mass Spectrom 18:2299–2308

Yager TJB, Furlong ET, Kolpin DW, Kinney CA, Zaugg SD, Burkhardt MR (2014) Disspiation of contaminants of emerging concern in biosolids applied to nonirrigated farmland in eastern Colorado. J Am Water Resour Assoc 50:343–357

Yan Z, Liu X, Yuan Y, Liao Y, Li X (2013) Deodorization study of the swine manure with two yeast strains. Biotechnol Bioprocess Eng 18:135–143

Yasuhara A (1987) Identification of volatile compounds in poultry manure by gas chromatography-mass spectrometry. J Chromatogr 387:371–378

Yin B, Gu J-D (2006) Aerobic degradation of 3-methylindole by *Pseudomonas aeruginosa* Gs isolated from mangrove sediment. Hum Ecol Risk Assess 12:248–258

Yin B, Huang L, Gu J-D (2006) Biodegradation of 1-methylindole and 3-methylindole by mangrove sediment enrichment cultures and a pure culture of an isolated *Pseudomonas aeruginosa* Gs. Water Air Soil Pollut 176:185–199

Yokoyama MT, Carlson JR (1979) Microbial metabolites of tryptophan in the intestinal tract with special reference to skatole. Am J Clin Nutr 32:173–178

Zhang W, Wei C, Yan B, Feng C, Zhao G, Lin C, Yuan M, Wu C, Ren Y, Hu Y (2013) Identification and removal of polycyclic aromatic hydrocarbons in wastewater treatment processes from coke production plants. Environ Sci Pollut Res 20:6418–6432