RESEARCH ARTICLE

A novel bacterium involved in the degradation of 2-methylindole isolated from sediment of Inner Deep Bay of Hong Kong

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Abstract: A bacterial strain, designated as MPKc, was isolated from the mudflat sediment of Mai Po Inner Deep Bay of Hong Kong Mai Po Nature Reserve by enrichment culturing with 2-methylindole as the sole source of carbon and energy. The microorganism was a Gram-negative, rod-shaped (0.4–0.6 μm × 1.0–2.2 μm) and aerobic bacterium. Phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain MPKc should be assigned as a novel bacterium, at least, at the species level. The 16S rDNA sequence most similiar to that of strain MPKc was *Azoarcus evansii* (94%) from available 16S rDNA sequences of the GenBank, indicating that strain MPKc was a member of the *β*-subclass of the *Proteobacteria*. Biochemical tests showed that strain MPKc was able to reduce nitrate to nitrogen. Carbon sources utilized by this strain included adipic acid, malate, citrate and phenylacetic acid although it only grew weakly on glucose, arabinose, mannose, mannitol, *N*-acetyl-glucosamine, maltose and gluconate. Strain MPKc showed no growth on capric acid. Its optimal growth occurred at 30°C, pH 6.5–7.5 and salinity 5–10‰. Strain MPKc was capable of degrading 80 μM 2-methylindole in 7 days under aerobic conditions. The possible chemical pathway for 2-methylindole degradation is through oxidation at 3-position or/and 2-position of the pyrrole ring.

Keywords: 3-methylindole, metabolism, Mai Po Nature Reserve, culturability

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1. Introduction

Indolic compounds, including indole, 1-methylindole, 2-methylindole and 3-methylindole, are nitrogenous heterocyclic aromatics. They are ubiquitous in our environment because of their common presence in coal tar[1], cigarette smoke[2] and animal wastes[3,4] and also their wide usage in industries for making dyes, medicine, pesticides, agricultural chemicals and industrial solvents[5–7]. Although their toxicity has not been well documented in details, Wilkes[8] and Ochiai *et al.*[9] have suggested that they are potentially carcinogenic, posing a serious threat to humans and animals. Ochiai *et al.*[9] studied the mutagenicities of indole and its 30 derivatives, including 1-methylindole, 2-methylindole and 3-methylindole, after nitrite treatment because nitrite is taken into human body and also formed endogenously and it reacts with various compounds to yield mutagens. They found that, among 31 tested indole derivatives, 1-methylindole showed the highest mutagenicity after nitrite treatment to *Salmonella typhimurium* TA100. They

also concluded that the mutagenic precursor activities of both 1-methylindole and 2-methylindole are even comparable with the mutagenicities of typical carcinogens such as benzo[a]pyrene and 3-methylcholanthrene[10]. Due to their wide presence and potential hazards in the environment, it is significant to study their fates so as to evaluate their risks in the environment. It is widely believed that bacteria play a vital role in transforming chemicals in the ecosystem and facilitating nutrient cycling. Therefore, studying their biodegradation by bacteria is essential for understanding their fates and evaluating their hazards in the environment.

Despite the great importance of studying the biodegradation of indolic compounds, information on the degradation, transformation and the fate of these chemicals, especially 1-methylindole and 2-methylindole, is fairly limited[7]. To my best knowledge, up to now, only Gu and Berry[11], Johansen *et al.*[12] and Gu *et al.*[7] have studied the biodegradation of 1-methylindole and 2-methylindole. All of these studies were carried out under strictly anaerobic conditions, namely methanogenic or sulfate-reducing conditions, and none of them demonstrated that bacteria could degrade 1-methylindole or 2-methylindole. In contrast, degradation of 3-methylindole has been reported under methanogenic[7,11,13] and sulfate-reducing conditions[7,12].

The Mai Po and Inner Deep Bay wetland, located at the border of the north-western part of Hong Kong Special Administrative Region, People's Republic of China and of the south-western part of the Shenzhen Special Economic Zone in the Guangdong Province of China, was listed as a Wetland of International Importance under Ramsar Conservation in September 1995. It is the largest remaining wetland in Hong Kong and plays a very important role in supporting a wide range of wild life including migratory birds and local important species[14]. It is not only an important breeding, feeding, resting and refuelling station in winter for over 250 species of birds[15] including 12 rare and threatened species of birds[16], but also supports a diverse community of local flora and fauna, including over a dozen endemic invertebrate species[14,15]. In recent years, the Ramsar site especially the mudflat of the Inner Deep Bay is subjected to the increasing pollution pressure from the rapid economic development on both sides of the Shenzhen River and the increasing pollution loads from the Pearl River, the Shenzhen River, the Yuen Long Creek and the Kam Tin River[17,18].

Recently, environmental *Vibrio* species have been isolated and identified from this area readily and the isolates showed various characteristics in responding to environmental conditions as recently tested in this laboratory[19–21]. In addition, these bacteria have a frequency of 8% bearing plasmids, implicating for the resistance to a range of chemicals and other unknown biological function[22]. Due to the constant discharge from the rivers, the mudflat sediment of Inner Deep Bay may harbour different groups of pollutant-degrading microorganisms as demonstrated with the endocrine-disrupting phthalate esters[23–26]. Therefore, the sediment of the mudflat of Inner Deep Bay was chosen to be the inoculum in this study. The objectives of this study were to isolate the 2-methylindole-degrading bacteria from the mudflat sediment of Inner Deep Bay, and to characterize the isolated bacterium on the basis of morphology, physiology and biochemistry.

2. Materials and Methods

2.1 Enrichment, Isolation, Purification and Preservation of Microorganisms

The microorganism was isolated with enrichment culturing technique using the sediment from the mudflat of Mai Po Inner Deep Bay of Hong Kong SAR as an inoculum and 2-methylindole as the sole source of carbon and energy. The mineral salts medium (MSM) used for the initial enrichment culture and several successive transfers consisted of: 0.8 g/L K_2HPO_4; 0.2 g/L KH_2PO_4; 1 g/L $(NH_4)_2SO_4$; 0.05 g/L $CaCl_2$; 0.5 g/L $MgCl_2{\cdot}6H2O$; 0.01 g/L $FeCl_3{\cdot}6H_2O$; 18 g/L NaCl. The medium was adjusted to pH 7.5 ± 0.1 with dilute NaOH or HCl and autoclaved at 120°C, at 2 atm for 15 minutes. The initial enrichment culture was established by adding approximately 2 g mudflat sediment into a 250 mL Erlenmeyer flask containing 100 mL of pre-sterilized MSM and 0.1 mL of 100 mM 2-methylindole (Acros Organics, USA, purity, 98%) solution dissolved in methanol after passing through a cellulose acetate syringe filter of 0.2 μm pore size (IWAKI Glass, Japan). The cultures were incubated in an incubator shaker (New Brunswick Scientific, USA) at 150 rpm and 30 ± 0.5°C. The experimental setup included duplicate and one control.

During the experiment, 1 mL of aliquot sample was taken from each Erlenmeyer flask containing the enrichment cultures at regular time intervals (2-days) and filtered through 0.2 μm-pore-size-membrane syringe filter (IWAKI Glass, Japan) for analyzing the concentrations of 2-methylindole. The samples were

quantified by external standards method on High-Performance Liquid Chromatography (HPLC) (Agilent 1100 series, Agilent Technologies) as described below. When 2-methylindole disappeared, 5 mL of established microcosms was transferred from the initial enrichment culture into new Erlenmeyer flasks containing pre-sterilized MSM and 2-methylindole.

After three successive transfers were carried out, the bacterial cultures obtained were purified aseptically through spreading and streaking technique. The cultures were diluted and then inoculated on selective agar plates in duplicate. The selective agar plates consisted of MSM as described above, 0.1 mM 2-methylindole (Acros Organics, USA), 0.1% Nutrient broth (Oxoid Ltd., England) and 1.5% agar (Lab M, UK). After 4 days of incubation at 30°C, a number of tiny, well-separated, individual colonies with different morphological characteristics were developed and visible. Individual colonies with different morphological appearances were streaked onto fresh nutrient (Oxoid Ltd., England) agar (Lab M, UK) plates and incubated at 30°C for 3 days. The streaked plates were assessed for purity based on colony morphology and microscopic observation. Re-streaking was carried out when it is necessary to achieve pure culture of the isolated bacteria.

After the pure cultures were achieved, they were preserved using glycerol. 0.85 mL of pure cultures grown in nutrient broth medium (Oxoid Ltd., England) were added aseptically into sterile cryovials with 0.15 mL of sterilized glycerol each and were stored frozen at −70°C as the Bacterial Culture Collection deposited in the Laboratory of Environmental Toxicology.

2.2 Identification of 2-Methylindole-degrading Bacterial Isolate

Smear Preparation and Gram Staining

The pure culture of the bacterial isolate strain MPKc capable of degrading 2-methylindole was Gram-stained[27]. Heat-fixed culture mounts on glass slide were stained with crystal violet (primary stain) for 20 s. After the stain was washed off with distilled water gently, the smears were covered with Gram's iodine solution for 1 min. Afterwards, the smears were washed with 95% ethyl alcohol for 20 s to decolourize the Gram-negative bacteria. Safranin, a counter-stain, was added to the smears for 20 s and then excess stain was washed off gently with distilled water. The Gram stained fixed mounts were observed under light microscope to determine the Gram stain reaction and assess the cellular morphological features.

Physiological and Biochemical Characteristics

The biochemical characteristics of the bacterial isolate strain MPKc were investigated using the API 20NE Multitest Kit (bioMérieux, France) following the instructions as described in the manufacturer's menu (bioMérieux 07615F, 2001). The biochemical results obtained were used to establish possible identification with similarity to those in the database by using Profile Index (bioMérieux, 1997). The detection of the enzyme cytochrome oxidase was performed with oxidase reagent (bioMérieux, France) on a filter paper. The ability of strain MPKc to reduce sulfur-containing compounds, sodium thiosulfate, to hydrogen sulfide was also tested. A tube of a semi-solid agar medium supplemented with 0.02% sodium thiosulfate and 0.015% ferrous sulfate[28] was inoculated with liquid culture of the strain MPKc by streaking.

16S rDNA Sequencing

Total genomic DNA of the bacterium strain MPKc was extracted using DNeasy® tissue kit (Qiagen Inc., USA) following the instructions as described in the handbook of the kit. 1.5 mL culture of the bacterium grown in nutrient broth (Oxoid Ltd., England) incubated in an incubator shaker (New Brunswick Scientific, USA) at 150 rpm and 30°C for 48 hours were centrifuged at 7500 rpm for 10 min in a microcentrifuge tube. After discarding the supernatant, the pellet was re-suspended in 180 µL Buffer ATL. Then, the tissue in the suspension was completely lysed with 20 µL proteinase K at 55°C. 200 µL Buffer AL was then mixed with the samples thoroughly by vortexing and incubated at 70°C for 10 min. After mixing the sample with 200 µL ethanol (96–100%) by vortexing, the mixture was centrifuged at 8000 rpm for 1 min in the DNeasy Mini Spin Column with a 2 mL collection tube. Afterward, the sample was centrifuged with 500 µL Buffer AW1 in the column with a new 2 mL collection tube at 8000 rpm for 1 min. In order to ensure that no carryover of residual ethanol occurs, 500 µL Buffer AW2 was added into the sample and centrifuged for 3 min at 14000 rpm in the column with a new collection tube. The DNeasy Mini Spin Column was placed in a clean 1.5 mL microcentrifuge tube followed by direct addition of 200 µL Buffer AE onto the DNeasy membrane, keeping at room temperature for 1 min and the centrifuging for 1 min at 8000 rpm subsequently. Such step was repeated again without replacing the column

into a new microcentrifuge tube.

After the extraction of the genomic DNA of the bacterium strain MPKc, 16S rRNA gene was amplified with the universal primers forward: pA (5'-AGAGTTTGATCCTGGCTCAG-3'; *E. coli* bases 8 to 27) and reverse: PC5B (5'-TACCTTGTTACGACTT-3'; *E. coli* bases 1507 to 1492)[29]. Amplified reaction mixtures contained 5 µL of deoxynucleoside triphosphate mixture (20 mM), 5 µL of 10×Taq DNA polymerase buffer, 6 µL of $MgCl_2$ (25 mM), 2 µL of each primer (25 pmol/µL), 3 µL of DNA template (20 ng/µL), 0.5 µL of Taq polymerase (5 U/µL) in a final reaction volume of 50 µL. Polymerase Chain Reaction was conducted with a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Massachusetts, USA) as follows: 2 min of the denaturation at 94°C, followed by 30 cycles of 30 sec at 94°C (denaturation), 30 sec at 50°C (annealing), and 60 sec at 72°C (extension), with a final extension at 72°C for 5 min after 30 cycles were completed.

The amplified product was purified using Qiagen PCR Purification kit and the 1.5 kb fragment was ligated into the pGEM-T Easy vector (Promega Corporation, Madison, Wisconsin, USA) by using T4 DNA ligase. The total volume of 10 µL ligation mixture, containing 2 µL of DNA fragment, 1 µL of 50 ng/µL pGEM-T vector, 2 µL of 5 × T4 DNA buffer, 0.5 µL of 3U/µL T4 DNA ligase and 4.5 µL of distilled water, was incubated at 16°C overnight. The product of ligation was then transformed into competent *E. coli* JM109 cells as described elsewhere[30]. 2 µL of the product from ligation was mixed with 200 µL of suspension of competent *E. coli* JM109 cells in a sterile microfuge tube by swirling gently and then was kept in ice for 30 min. After kept the tube in a water bath at 42°C for exactly 90 seconds, the cells were chilled in an ice bath for 1–2 minutes. The culture with 800 µL SOC medium (2% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose, at pH 7.0) was incubated in a water bath at 37°C for 45 minutes to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. Then, 100 µL of transformed competent cells was transferred into agar LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1.5% agar and 1% NaCl at pH 7.5) containing 100 µg/mL amplicillin, 40 µg/mL X-gal (5-bromo-4-chloror-3-indolyl-β-D-galactoside) and 40 µg/mL IPTG (isopropylthio-β-D-galactoside). After incubation at 37°C for 12 hours, the positive colonies, identified by blue-white color selection on agar plates, were selected and grew on LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl at pH 7.5) supplemented with 100 µg/ml amplicillin overnight.

The liquid culture of the positive colonies stored in a sterile microcentrifuge tube was sent to the Takara Biotechnology (Dalian) Co. Ltd. (Dalian, P.R. China) for extracting the recombinant plasmid from the cells and determining the 16S rRNA gene sequence. The inserted fragment was sequenced with an ABI Prism 377XL DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, California), initially by using pGEM-T vector specific primers, then the internal primer.

The 16S rDNA sequence was aligned with related sequences from GeneBank using BioEdit Sequence Alignment Editor (BioEdit version 5.09, Department of Microbiology, North Carolina State University, U.S.). The phylogenetic and molecular evolutionary analyses were conducted with MEGA (Molecular Evolutionary Genetic Analysis) program package, version 2.1 (http://www.megasoftware.net/) by using the neighbour-joining method[31].

Scanning and Transmission Electron Microscopy

Culture of the strain MPKc was fixed on a 0.2 µm-pore size polycarbonate membrane filter (Osmonics, Livemore, California) in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer overnight. The specimens were then prepared for SEM examination following the procedures of initially washing with 0.1 M sodium cacodylate three times for 2 min each rinse, further fixing with 1% osmium tetroxide in 0.1 M sodium cacodylate for 8 hours, rinsing with sodium cacodylate buffer and distilled water, dehydrating with ethanol series of 40% to 80% ethanol in 10% increments and then 80% ethanol to 100% ethanol in 5% increments, critical-point drying using liquid carbon dioxide and finally coating with gold-palladium as described elsewhere[32]. The prepared samples were observed under a Leica Cambridge S440 scanning electron microscope.

The culture of strain MPKc was also prepared for TEM examination by first fixing the cell suspension in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 hour at 4°C; re-suspending the cells subsequently in cacodylate with 0.1 M sucrose and in cacodylate buffer; and then further fixing the cell pellet in 1% osmium teroxide in cacodylate buffer for 30 min at room temperature, followed by centrifuging the cell pellet with equal volume of 2% agar immediately

at 2500 rpm for 10 min for the producing the gel block. Afterward, the gel block cut into 1 mm cubes was dehydrated with ethanol series of 50%, 70% and 90% for 5 min each and then 3 times of 100% for 10 min each and 2 times of propylene oxide for 5 min each. After infiltrating the tissue blocks subsequently with the mixture of epoxy resin and propylene oxide (1:1; v:v) for 1 hour 30 min at 37°C and epoxy resin for 1 hour at 37°C, the tissue blocks were embedded in epoxy resin and polymerized at 90°C for 2 hours. Semi-thin sections in 0.4 μm thickness were cut with a Reichert ultramicrotome and stained with 0.5% toluidine blue in 1% sodium borax for 30 sec. Ultra-thin sections in 100 nm thickness were further cut from the semi-thin sections with the target elements confirmed with light microscope in a Reichert ultramicrotome and stained subsequently with 2% (w/v) uranyl acetate for 20 min and lead citrate for 15 min. The prepared sections were then observed under a Hitachi H-600 transmission electron microscope.

2.3 Characterization of Bacterial Isolate

Effects of Temperature

100 mL Erlenmeyer flasks containing 50 mL of freshly prepared sterile nutrient broth (Oxoid Ltd., England) were inoculated with 0.1 mL liquid culture of strain MPKc grown in nutrient broth incubated in an incubator shaker (New Brunswick Scientific, USA) at 150 rpm and 30°C for 36 hours. Flasks were incubated in water-bathes with shaking (Julabo SW20, Japan) and adjustable cooling system (Julabo FT200, Japan) at 15°C and 22°C at 150 rpm and incubator shakers (New Brunswick Scientific, USA) at 30°C and 40°C at 150 rpm. Each temperature treatment was in duplicate. Flasks containing non-inoculated sterile nutrient broth were used as blank controls. During the incubation, optical density was measured at 600nm (OD_{600}) spectrophotometrically using UV-1201V Spectrophotometer (Shimadzu, Japan).

Effects of pH

100 mL Erlenmeyer flasks containing 50 mL of freshly prepared sterile nutrient broth (Oxoid Ltd., England) with four different pH values (pH 5.5, 6.5, 7.5 and 8.5) adjusted with NaOH or HCl were inoculated with 0.1 mL liquid culture of strain MPKc as mentioned above. Flasks were incubated in an incubator shaker (New Brunswick Scientific, USA) at 30°C and 150 rpm. Each pH treatment was in duplicate. Four flasks containing non-inoculated sterile nutrient broth with four different pH values were used as blank controls. During the experimental period, OD_{600} values for all flasks were measured with UV-1201V Spectrophotometer (Shimadzu, Japan).

Effects of Salinity

Nutrient broth (Oxoid Ltd., England) with different salinity (2.5, 5, 10, 15, 20 and 30‰) were prepared by mixing with equal amount of distilled water and appropriate amount of analytical grade NaCl before autoclaving. Then, 0.1 mL liquid culture of strain MPKc was added into each flask containing 50 mL sterile nutrient broth of different salinity prepared as mentioned above. Flasks were incubated in an incubator shaker (New Brunswick Scientific, USA) at 30°C and 150 rpm. Non-inoculated blank controls were also prepared. The experimental set up included duplicate for each treatment. During the experimental period, OD_{600} values for all flasks were measured with UV-1201V Spectrophotometer (Shimadzu, Japan).

Analysis of Growth Data with the Gompertz Model

The Gompertz model (Equation 1), which was found to be the most robust model to fit the bacterial growth data and easy to use[33] among many developed models for describing the bacterial growth curve[34,35], was applied in the calculation of three parameters, the specific growth rate (μ_m), lag time (λ) and maximum biomass reached (A), during the bacterial growth under different studied conditions as described elsewhere[21,36].

$$X = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\} \qquad [1]$$

where X is the biomass concentration; t is the time of incubation (hour); μ_m is the maxmium specific growth rate ($hour^{-1}$); A is the maximum biomass reached; λ is the lag time (hour); e is a constant, 2.718281828. The above nonlinear equation was used to fit the biomass accumulation data using nonlinear least square regression method. All data analysis was performed using Matlab 6.0 with Optimization Toolbox 2.1 (The Mathworks Inc, 2000) for minimizing the residual sum of squares (RSS) with Levenberg-Marquardt algorithm. Levenberg-Marquardt algorithm has been successfully tested on a large number of nonlinear problems and has proved to be more robust than the Gauss-Newton method and iteratively more efficient than an unconstrained method. The R-square value (R^2) and the residual sum of squares (RSS) are indices indicating

how fit the model to the experimental data.

The Plasmid DNA

Strain MPKc was also screened for the existence of plasmid. 2 mL of the liquid culture of strain MPKc grown in nutrient broth medium (Oxoid Ltd., England) incubated in an incubator shaker (New Brunswick Scientific, USA) at 30°C and 150 rpm for 48 hours was harvested by centrifuging at 12000 rpm for 3 min and then discarding the supernatant. The plasmid DNA was then prepared by following the protocol modified from the one described by Sambrook and Russell[30].

Firstly, the bacterial pellet was re-suspended in 100 μL of ice-cold Solution I (50 mM glucose, 25 Mm Tris·Cl at pH 8.0 and 10 Mm EDTA at pH 8.0) by vigorous vortexing. The tube was inverted gently for several times after the addition of 200 μL of freshly prepared Solution II (0.2 M NaOH and 1% SDS) before being stored in an ice-bath. 150 μL of ice-cold Solution III (60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of distilled water) was introduced. After the tube was kept in the ice-bath for 4 min, it was centrifuged at 14000 rpm for 15 min and the supernatant was transferred into a new centrifuge tube. The double-stranded DNA in the supernatant was precipitated with 0.6 volumes of propan-2-ol at room temperature by vortexing. The tube was air-blow dried after centrifuging (5 min × 12000 rpm) and carefully discarding the supernatant. Afterwards, the nucleic acid was re-dissolved in 50 μL of TE (pH 8.0). The resulting DNA preparation was then examined by electrophoresis using 0.8% agrose gel consisting of the gel buffer (40 mM Tris-Acetate and 1 mM Ethylenediaminetetraacetic acid, EDTA) and 0.5 μg/mL Ethidium Bromide (EtBr).

The extraction and preparation of plasmid from strain MPKc and the electrophoresis for the resulting DNA preparation were conducted for the second time to further confirm the negative results of plasmid in strain MPKc. In parallel, plasmids were extracted successfully from plasmid-positive bacteria.

Degradation of 2-methylindole

The experiment was carried out in 250 mL Erlenmeyer flasks containing 100 mL of pre-sterilized MSM as described and 80 μM 2-methylindole (Acros Organics, USA, purity, 98%). The 0.1 mL sterile 2-methylindole solution added to each flask was prepared by firstly dissolving 2-methylindole solute (Acros Organics, USA, purity, 98%) in 1mL HPLC grade methanol and then passing through a 0.2 μm-pore-size cellulose acetate membrane syringe filter (IWAKI Glass, Japan). This experiment included two treatments, with the inoculation of active strain MPKc and autoclave-killed (inactivated) strain MPKc respectively, in duplicate, and one control without inoculation. For the treatment with the inoculation of active strain MPKc, 0.1 mL of culture of strain MPKc grown in nutrient broth medium (Oxoid Ltd., England) in a shaker incubator (New Brunswick Scientific, USA) at 150 rpm and 30 ± 0.5°C for 48 hours was used as inoculum for each flask. For the treatment with the inoculation of inactivated strain MPKc, 1 mL of the culture of live strain MPKc mentioned above was autoclaved for 20 min and then 0.1 mLof the autoclaved culture was then used as the inoculum for each flask. All flasks were incubated in an incubator shaker (New Brunswick Scientific, USA) at 150 rpm and 30 ± 0.5°C.

During the experiment, the change in the concentration of 2-methylindole in the flasks was monitored by withdrawing 1.0 mL of aliquot from each Erlenmeyer flasks at daily intervals and filtering through 0.2 μm-pore size cellulose acetate membrane syringe filter (IWAKI Glass, Japan). All samples were quantified by external standard methods using High-Performance Liquid Chromatography (HPLC) (Agilent 1100 series, Agilent Technologies) as described below immediately after sampling.

Analysis of Substrate Compound

The filtrates of samples were quantified by using High-Performance Liquid Chromatograph (HPLC) system (Agilent 1100 series, Agilent Technologies) consisting of a quaternary low-pressure degasser, a quaternary high-pressure pump, a model 7725i manual sample injector with a 20 μL sample loop, and diode array and multiple wavelength detectors. Separation of 2-methylindole was accomplished by using a 4.6 × 150 mm Eclipse 5-μm XDB-C8 reversed-phase liquid chromatography column. A mixture of methanol and water (60:40, vol/vol) delivered at a flow rate of 1 mL min^{-1} was used as a mobile phase. The total dilution time for each sample was approximately 6 min. The 2-methylindole in samples was quantified by external standards method at wavelength of 270 nm. The calibration curve was linear for 2-methylindole (Acros Organics, USA, purity, 98%) in the range from 0 μM to 1000 μM (R^2 = 0.99996). The UV-visible spectra were recorded at identical retention time to confirm the

identification of the compound.

3. Results and Discussion

3.1 Isolation of Microorganism

Based on the morphology of bacterial colony visually and cells microscopically, a total of 29 different bacterial isolates were obtained from the enrichment cultures. The isolate, strain MPKc, capable of degrading 2-methylindole, was selected from them for subsequent investigation in this study because of its most effectiveness.

3.2 Identification of Strain MPKc

Under light microscope, cells of strain MPKc appeared as rods in singly and mainly in pairs and chains. From the scanning electron micrograph (Figure 1A), the size of the cells of strain MPKc were 0.4–0.6 μm in width and 1.0–2.2 μm in length and the extracellular polysaccharides, which looks like fibers on the surface of cells, could also be seen. They were motile. Spores were not observed. Strain MPKc was Gram-negative. The transmission electron micrograph (Figure 1B) also showed the cells of strain MPKc possessed a typical Gram-negative cell wall structure.

Based on the full sequence (1495 nucleotides) of the 16S rRNA gene of strain MPKc, a phylogenetic tree was constructed by comparing the 16S rDNA sequence of strain MPKc and that of closely related sequences deposited in GenBank using neighbour-joining method[31] (Figure 2). The nearest phylogenetic neighbour of strain MPKc was a bacterium (sequence similarity 100%) with the accession number, AB049763, in the GenBank. Although a proposed name for that bacterial species has been deposited in the GenBank, none of the paper for its valid name and any information about that species have been published. Besides the mentioned, unpublished one (sequence accession number AB049763, similarity 100%) and the uncultured bacterial clone PL-33B2 (sequence accession number AY570620, similarity 95%), it was mostly closely related to *Azoarcus evansii* (sequence accession number X77679, similarity 94%), suggesting strain MPKc should belong to the family *Rhodocyclaceae* in the *β*-subclass of the *Proteobacteria*. However, such low sequence similarity suggests that the isolate strain MPKc should be assigned as a novel bacterium, at least, at the species level.

3.3 Physiological and Biochemical Characteristics

The physiological and biochemical characteristics of strain MPKc were summarized in Table 1. Strain MPKc was an aerobic bacterium and gave positive results to the oxidase test. Physiological tests revealed that the isolate strain MPKc showed the following activities. Neither indole production from tryptophan nor hydrogen sulfide production from thiosulfate was observed; glucose fermentation; arginine dihyrolase; urease; β-glucosidase, protease and β-galactosidase were negative. Strain MPKc reduced nitrates to nitrogen. Strain

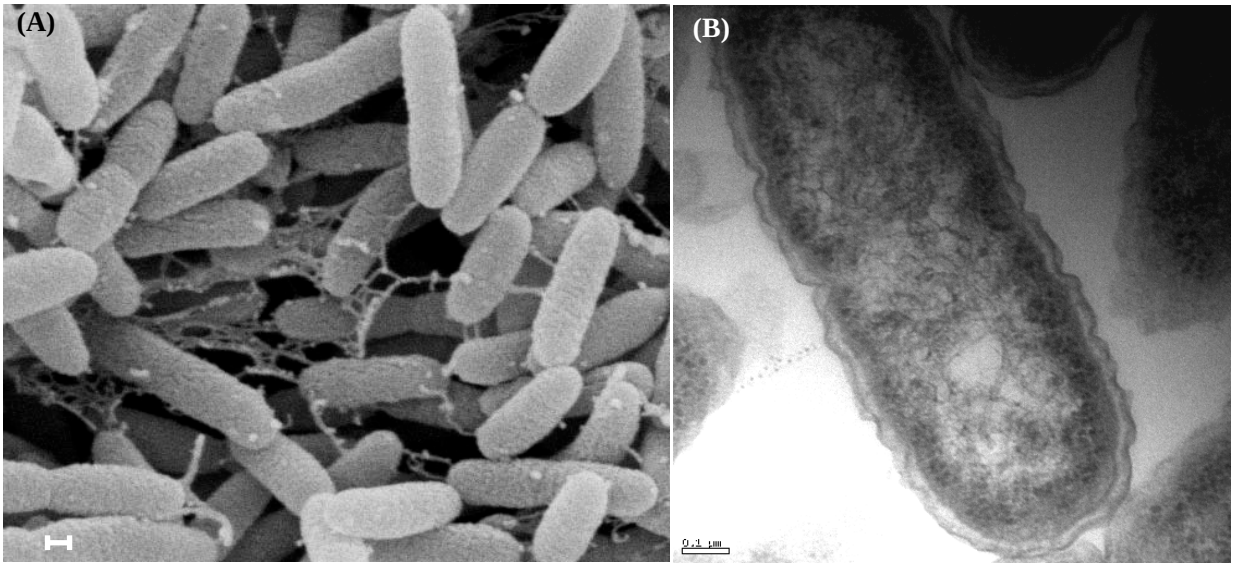

Figure 1. (A) A scanning electron micrograph of strain MPKc on a membrane filter, Bar , 200 nm. **(B)** A transmission electron micrograph of strain MPKc cells showing the typical cell wall structure of Gram-negative bacteria. Bar, 0.1 μm.

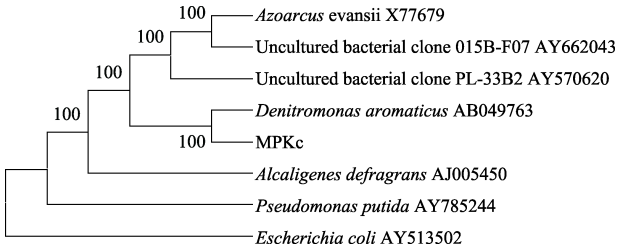

Figure 2. Phylogenetic tree of strain MPKc capable of degrading 2-methylindole isolated from sediment of Inner Deep Bay Ramsar Site of Hong Kong and related microorganisms based on their 16S rDNA sequences. Bootstrap frequency values are given at branching points of interest.

Table 1. Biochemical characteristics of the isolated strain for degradation of 2-methylindole

Characteristics	Responses
Nutrient agar colonies	Positive
Nitrate reduction to nitrogen	Positive
Indole production	Negative
Acidification	Negative
Arginine dihydrolase	Negative
Urease	Negative
Hydrolysis (β-glucosidase)	Negative
Hydrolysis (protease)	Negative
β-galactosidase	Negative
Hydrogen sulfide production	Negative
Oxidase test	Positive
Utilization of carbon compounds	Grown on adipic acid, malate, citrate and phenylacetic acid
	Very weak growth on glucose, arabinose, mannose, mannitol, maltose, gluconate and N-acetyl-glucosamine
	No growth on capric acid
Oxygen demand	Aerobic

MPKc showed a wide spectrum in the utilization of various carbon sources. It grew on adipic acid, malate, citrate and phenylacetic acid, however, it only grew weakly on glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose and gluconate. No growth was observed on only one carbon source, capric acid, among those tested in this study.

3.4 More Characteristics of Strain MPKc

Effect of Temperature, pH and Salinity on the Bacterial Growth

The effect of temperature, pH and salinity on the growth of Strain MPKc, in terms of maximum specific growth rate (μ_m), lag time (λ) and maximum biomass (A), is shown in Table 2. High values of R^2 (>0.94) and small values of RSS (<0.05) for all data sets indicated the applicability and the robustness of the Gompertz model for data analysis in this study.

Strain MPKc was very sensitive to the temperature change. The results demonstrated an increase in specific growth rate and a decrease in lag time of strain MPKc from low temperature, 15°C, to high temperature, 30°C. However, the maximum specific growth rate at 40°C declined drastically. Strain MPKc showed optimal growth at 30°C with the highest specific growth rate among four tested temperature treatments and a shorter lag time compared with that at 22°C. Although the lag time for strain MPKc was shortest, among all four temperature treatments, at 40°C, strain MPKc could only grow slowly (0.0123 hr^{-1}) and could not reach the maximum biomass as high as those at other temperatures. It is not surprising that strain MPKc showed almost lowest specific growth rate and the longest lag time, among all four temperature treatments, at 15°C because of the low enzyme activity as expected.

Concerning the growth of strain MPKc at different pH, although the specific growth rates of strain MPKc were similar at pH 5.5, 6.5 and 7.5, its lag phase at pH 5.5 was nine hours longer than that at pH 6.5 and 7.5, indicating that strain MPKc was more sensitive to lower pH. Therefore, pH 5.5 was not an optimum pH for the growth of strain MPKc. The shortest lag time and the highest specific growth rate of strain MPKc at both pH 6.5 and 7.5 among four pH treatments demonstrated that strain MPKc grew optimally at pH 6.5–7.5. Furthermore, the strain MPKc could grow up to pH 9.5, indicating that the hydrogen ion concentration at pH 9.5 was too high that limited the activity of enzyme with which the bacterium could synthesize new protoplasm.

Besides temperature and pH, salinity is also a significant parameter in affecting bacterial growth in relation to the environment where this bacterium was isolated. Strain MPKc preferred to grow at relatively low salinity. Since strain MPKc possessing similar specific growth rate (0.05 hr^{-1}) at salinity 5‰ and 10‰, optimal growth was achieved at around salinity 5‰ to salinity 10‰ even though its lag time at 5‰ was slightly shorter than that at 10‰. Lower μ_m of strain MPKc and its longer λ comparing with other salinity treatments (2.5, 5, 10 and 15‰) demonstrated that the salinity higher than 20‰ started to show significant negative effect on the growth of strain MPKc.

Table 2. Specific growth rate (μ_m), lag time (λ) and maximum biomass yield (A) of the isolate strain MPKc under different temperature, pH and salinity using the Gompertz model

	Specific growth rate	Lag time	Maximum biomass	R^2	RSS
	μ_m±SD ($hour^{-1}$)	λ±SD (hour)	A±SD (OD_{600})		
Temperature (°C)					
15	0.0165±0.0001	42.5737±1.4368	0.8342±0.0291	0.9890	0.0196
22	0.0571±0.0016	18.5335±0.1241	0.7126±0.0069	0.9783	0.0459
30	0.1029±0.0028	11.0003±0.0285	0.6835±0.0029	0.9926	0.0195
40	0.0123±0.0050	3.6258±3.8365	0.5920±0.0666	0.9924	0.0061
pH					
5.5	0.1012±0.0040	18.8289±2.0838	0.6911±0.0151	0.9936	0.0191
6.5	0.1042±0.0038	11.0502±0.0431	0.6915±0.0023	0.9906	0.0245
7.5	0.0989±0.0073	10.9688±0.0733	0.6787±0.0102	0.9907	0.0231
8.5	0.0123±0.0008	14.8178±0.0226	0.5987±0.0173	0.9825	0.0240
9.5	ND	>70 hrs*	0.0000	ND	ND
Salinity (‰)					
2.5	0.0385±0.0027	7.2814±0.2194	0.3933±0.0105	0.9690	0.0193
5	0.0524±0.0005	7.4277±0.0824	0.4071±0.0004	0.9640	0.0252
10	0.0525±0.0003	8.2427±0.1867	0.4129±0.0014	0.9561	0.0346
15	0.0418±0.0032	8.9589±0.1783	0.4082±0.0050	0.9672	0.0274
20	0.0200±0.0007	10.0419±0.6322	0.3292±0.0503	0.9736	0.0080
30	0.0077±0.0008	18.2509±0.4459	0.4163±0.0652	0.9427	0.0163

* No bacterial growth observed during the whole period of experiment
ND, no data available (below detection limit)
SD, standard deviation of 2 replicates

The results also showed that salinity has less effect on the maximum biomass reached by strain MPKc when compared with temperature and salinity treatments.

Existence of Plasmid in Strain MPKc

Plasmids are extrachromosomal molecules of DNA in the size from 1 kb to more than 200 kb. They have been found in a wide variety of bacterial species and have been proved to be involved in various functions, including resistance to and production of antibiotics, degradation of complex organic compounds, and production of colicins, enterotoxins, and restriction and modification enzymes[30]. Recently, cryptic plasmids have been isolated from *Vibrio* spp. isolated from Mai Po Nature Reserve[18,21]. Because of this, it was important to investigate if plasmid was in strain MPKc. However, no plasmids was detected in this strain MPKc, suggesting that the functions of this strain were much more persistent because they did not rely on genes of plasmids for coding those enzyme.

Biodegradation of 2-methylindole

Initial 80 μM of 2-methylindole were completely degraded by the bacterial isolate strain MPKc in 7 days with 2-methylindole as the sole carbon source of carbon and energy (Figure 3). In contrast, there was no significant change in the concentration of 2-methylindole in sterilized controls during the whole experiments, indicating that the degradation of 2-methylindole due to natural hydrolysis and photolysis was negligible (Figure 3). Moreover, the constant concentration of

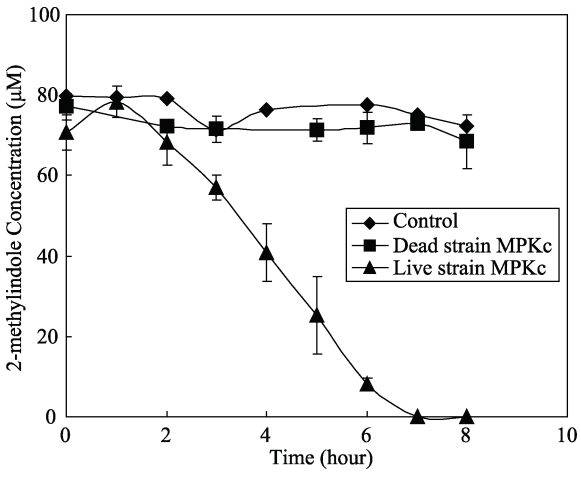

Figure 3. Degradation of 2-methylindole by the bacterial isolate strain MPKc isolated from sediment of Inner Deep Bay Ramsar Site of Hong Kong under aerobic conditions. Bar indicated standard deviation of duplicate.

2-methylindole in treatments inoculated with autoclave-killed culture of strain MPKc during the whole experiments further confirmed that the disappearance of 2-methylindole in treatments inoculated with live bacterial culture was not due to the adsorption on the bacterial cells (Figure 3).

Up to now, only Gu and Berry[11], Johansen *et al.*[12] and Gu *et al.*[7] have studied the biodegradation of 1-methylindole and 2-methylindole. Neither 1-methylindole nor 2-methylindole was demonstrated to be biodegradable under methanogenic conditions[7,11] and sulfate-reducing conditions[7,12]. It has been suggested that the methyl-group, which is an electron-donating substituent group, located at either 1- or 2- position would stabilize the indolium ion through direct inductive effects, leading to the inhibition of the attack of hydroxylation enzymes of bacteria stereochemically[7,11]. This study demonstrated that 2-methylindole could be degraded by strain MPKc at a low concentration. However, because of the high toxicity of 2-methylindole, strain MPKc could not tolerate high concentration of 2-methylindole. Less than 8% 2-methylindole disappeared within 8 days when the initial concentration of 2-methylindole was 0.3 mM (data not shown). Furthermore, since no plasmid DNA was found in strain MPKc as mentioned above, the degradation ability of strain MPKc for 2-methylindole should not be regarded as transferable metabolic ability under the selection pressure.

Biochemical mechanisms of indole degradation has been elucidated under strictly anaerobic conditions. Both methanogenic and sulfate-reducing conditions results in similar reaction mechanisms, mostly through initial hydroxylation and then dehydrogenation to introduce one oxygen atom onto the indolic substrate[7,11–13]. In contrast, little information is available for aerobic conditions. Given the fact that degradation reaction on heterocyclic aromatic compounds is always near the heteroatom through hydroxylation[37], the proposed biochemical pathway for 2-methylindole degradation by MPKc should be either at 3-position or oxidation of the methyl group at 2-position, the latter is more likely to be mediated by a monooxygenase if such reaction can be taken place (Figure 4).

Conclusion

In conclusion, the bacterial isolate, strain MPKc, isolated from the mudflat sediment, was involved in aerobic degradation of 2-methylindole. This study is the first report demonstrating that 2-methylindole was biodegradable so far. Strain MPKc should be considered as a new bacterium, at least, to the species level based on 16S rDNA sequencing. Strain MPKc did not have plasmid DNA and its optimal growth occurred at 30°C, pH 6.5–7.5 and salinity 5–10‰.

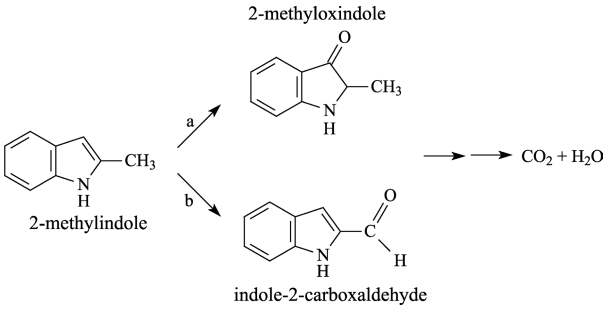

Figure 4. Proposed biochemical pathways for degradation of 2-methylindole by strain MPKc through oxidation of either (a) C-3 position or (b) the methyl group at 2-position.

Conflict of Interest and Funding

No conflict of interest has been reported by the authors.

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