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Isolation and characterization of marine sediment bacteria capable of biocatalyzing bromination of indole

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Abstract

The presence of naturally occurring halometabolites in the marine environment has led to studies focusing on the search for organisms capable of biocatalyzing halogenation reactions. In this study, we isolated two marine sediment bacteria (WK7 and WK12) from Mokpo, Jeollanam-do, South Korea with 99% identity to parts of the 16S rDNA of bacterial species Shewanella marisflavi and Pseudoalteromonas prydzensis, capable of biohalogenation activity based on the monochlorodimedone (MCD) assay. Isolate P. prydzensis WK12 (KP893638) produced compounds from indole with molecular ion peaks m/z 262.06 and 342.03 possibly corresponding to indigo and monobromoindigo. Likewise, S. marisflavi WK7 (KP893637) was speculated to have produced indigo and dibromoindigo (m/z 262.4 and 420.57) from indole. This is the first report on marine sediment bacteria capable of biocatalyzing bromination of indole as resting whole cells.

Introduction

In the earlier times, halogenated metabolites produced by microorganisms were thought to be uncommon in nature and it was only after 1968 that they were found to be quite abundant. Since 2012, it has been reported that over 5 000 naturally occurring organohalogen compounds are actually released into the environment by different organisms including bacteria, fungi, marine algae, lichens, higher plants, mammals, and insects[1]. One of the very first discovered halometabolites, chloramphenicol, was isolated from Streptomyces venezuelae. Different pharmaceutically important compounds were later discovered to be derived from other bacterial species. This reflects the fact that there is a great variety of halometabolites produced by bacterial cultures in different environment. The function of halometabolites in the environment is as diverse as their presence, varying from physiological to biological roles. It is hypothesized though that they are biosynthesized by organisms mainly for chemical defense and food gathering evolving from the stress of natural selection. Indeed, several halometabolites have shown defensive roles, specifically antibiotic activity (i.e., chloramphenicol and vancomycine). With this, the search for halometabolites produced by bacteria has always been connected to the search for novel antibiotics or other compounds capable of other biological activities. However, other industrially important natural products (agrochemicals) and commercially important compounds can also be derived from halometabolites or by themselves contain halogens[2].

This study aimed to isolate bacteria capable of brominating activity from marine sediments of Iro-dong, Mokpo, Jeollanam-do, South Korea. All isolates were screened using the monochlorodimedone (MCD) assay – the most widely used primary screening for haloperoxidases. Isolates that showed positive bromination activity were used as resting cell biocatalysts to check possible bromination of indole as a substrate. The formation of suspected halogenated products was checked using liquid chromatography/mass spectrometry (LC/MS).

Materials and methods

Bacteria isolation and growth conditions

Marine sediments were collected from Iro-dong, Mokpo-si, Jeollanamdo, South Korea (Latitude: 34°47'34.53"N, 34°47'34.27"N; Longitude: 126°24'43.84"E, 126°24'43.65"E). Ten grams of collected sediments was serially diluted using sterile phosphate buffer in 10-folds and the 10^{-5} dilution was plated in yeast extract tryptone sea salts (YTSS) agar, incubated for 24 h at 30ºC. YTSS agar contains 4 g yeast extract, 2.5 g tryptone, 20 g sea salts, and 18 g agar in 1 L of distilled water sterilized in 121ºC at 15 psi for 15 min[3]. A total of 200 individual colonies were picked out and purified five times or until a pure colony was obtained in the same medium and growth conditions. Isolates were stored in 50% glycerol at -60°C for further use. Routine phenotypical characterization of cells and colony of the isolates was also performed.

16S ribosomal DNA preparation and identification

Two selected isolates that showed possible brominating activity based on MCD assay were prepared for genomic DNA extraction. A 24 h culture (~2×10^9 cells) of each isolate was pelleted in a 1.5 mL microcentrifuge tube by centrifugation at 13 000 rpm for 1 min. The supernatant was discarded and the cells were resuspended in 180 µL of 30 mg mL^{-1} lysozyme and incubated at 37°C for 30 min. After incubation, 12 µL of 20 mg mL^{-1} of proteinase K was added to each mixture and the GeneAll® Exgene™ Cell SV mini kit (GeneAll Biotech., Seoul, Korea) was used following the manufacturer's instructions. Extracted DNAs were kept at -20ºC until further use.

For identification, the 16S rDNA was amplified using the universal primers, 27F and 1492R. Extracted DNA was prepared for amplification using the Accupower® PCR PreMix. The mixtures were amplified in the following conditions: initial denaturation step of 3min at 94ºC, 35 cycles of amplification for 30 s at 94ºC, 30 s at 55ºC, 1 min at 72ºC, and final extension step for 7 min at 72ºC using Long Gene® A300 Gradient Thermal Cycler. The PCR products were prepared for identification using the Bioneer AccuPrep®

PCR Purification Kit following the manufacturer's instructions. The purified PCR products were sent to Macrogen Inc., Seoul, South Korea for sequencing. All obtained nucleotide sequences were deposited to GenBank.

The phylogeny construction included the 16S rDNA sequences of two isolates. The statistical method used was Neighbor-Joining with Bootstrap method in 500 replications as a test of phylogeny. Evolutionary distances were obtained using Maximum Composite Likelihood as a model with substitution type set to nucleotide including transitions and transversions. Pairwise deletion method was used to treat gaps and missing data. The assignment of sequences, calculation of similarities between sequences, alignment of sequences, and evolutionary analyses were determined using BLAST and MEGA 6[4]. All strains included in the tree were obtained from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The outgroup used was Pseudomonas chlororaphis aureofaciens.

Monochlorodimedone (MCD) assay

A primary screening of isolates that can carry out bromination activity was done using the method employed by Moris and Hager[5]. Isolates were prepared by growing them in YTSS broth for 24 h in 30°C stirred at 200 rpm. The resting cells were collected by centrifugation at 6 000 rpm for 10 min in 4°C, washed with potassium phosphate buffer (pH 5.25 at 25°C), recentrifuged and were used directly as biocatalysts. The reaction mixture was based on the mixture optimized by Médici et al.[6] with the slight modifications. The mixture consisted of 0.1 M potassium phosphate buffer pH 5.25, 200 mM NaBr, 0.1 mM MCD (substrate), 8.8 mM hydrogen peroxide, and biocatalysts in a total volume of 3 mL. The reaction was initiated with the addition of the crude biocatalysts, incubated at 35°C for 2 h and stirred at 200 rpm[5]. The conversion of MCD to bromochlorodimedone was based on the decrease of MCD detected by the loss of absorbance of the mixture at λ=290 nm measured using a UV-spectrophotometer. The MCD assay was performed in triplicates.

Biotransformation reaction

Two MCD assay positive isolates were further screened using indole as substrate. The reaction mixture contains the following: Stanier's buffer, 1 mM glucose, 1.4 mM indole dissolved in methanol, 1.4 mM NaBr, 2 mM hydrogen peroxide (pulse addition every 2 h for 24 h), and the crude biocatalyst[5]. The isolates were prepared from a 24h YTSS culture (OD_{600}=6). The cells were pelleted by centrifugation in 4ºC at 6 000 rpm for 10 min. Pellets were washed three times and finally resuspended in Stanier's buffer. The reaction mixture was incubated in 30ºC for 24 h stirred at 200 rpm. Two controls were used throughout the experiment: (A) Bacterial control contains the isolates in Stanier's buffer, 1 mM glucose, 1.4 mM NaBr, and 2 mM H_2O_2 and (B) Substrate control contains Stanier's buffer, 1 mM glucose, 1.4 mM indole, 1.4 mM NaBr, and 2 mM hydrogen peroxide. The incubation time and condition were the same as mentioned above. All biotransformation activities were done in triplicates.

Analysis of reaction mixtures

To prepare samples for analysis, the 24 h reaction mixtures were extracted by adding ethyl acetate (1:3) and vigorously vortexing the mixture. When a distinct separation was achieved, supernatant was collected and concentrated using speed vacuum evaporator (Thermo Scientific Savant SPD 2010 SpeedVac® Concentrator) for 2 h in 75 psi. The concentrates from indole as substrate were dissolved in 1 mL methanol, filtered using sterile syringe 0.2 μm Grace® polyvinylidene (PVDF) filter and used directly for analysis. The analysis of new metabolites was performed using Alliance 2695 Waters with a Quattro LC triple quadruple tandem mass spectrometer (Waters) in an electrospray ionization (ESI) mode. The UV detection was set in PDA detector 2996 (Waters). The following conditions based from Puchalska et al.[7] were used: the mobile phase consisted of acetonitrile (A) and water with 0.1% (v/v) formic acid (B). A linear gradient program of 0→3 min from 0→20% A/80→20% B, 3→8 min from 30→50% A/70→50% B, 8→10 min from 50→55% A/50→45% B, 10→22 min in 55%A/45% B, 22→35 min from 55→100% A/45→0% B, and 35→45 min in 100% A/0% B at a flow rate of 0.6 mL min^{-1}.

Results and Discussion

Bacteria isolation and characterization

From a total of 200 colonies, only 19 purified isolates showed possible biohalogenating activity based on the MCD assay. The MCD assay was used as a primary screen to narrow potential isolates with haloperoxidases. The method is participated by a synthetic compound, monochlorodimedone (2-chloro-5,5-dimethyl-1,3-cyclohexanedione), where haloperoxidases catalyze its conversion to dichlorodimedone or bromochlorodimedone. The activity of haloperoxidase is measured by a decrease or loss of absorbance, assumed to be directly proportional to the formation of the compound[7]. In this experiment, the reaction mixture was primarily optimized to detect activity of haloperoxidases – enzymes that catalyze halogenation of compounds in the presence of halide ions and hydrogen peroxide. The most common and general mechanism of haloperoxidase involves their reduction of H_2O_2 to water and oxidizing a co-factor or a metal. The oxidized co-factor/metal reacts to the halogen and a proton to form an oxidized enzyme intermediate known as the hypohalous acid. The hypohalous acid, freely diffusible, is released from the enzyme to halogenate substrates. In cases where the haloperoxidase is metal- or co-factor free, formation of hypohalous acid intermediates is accomplished by the presence of a catalytic triad of amino acids[8].

Two isolates (WK7 and WK12) that yielded the fastest decrease in absorbance at λ=290 nm were identified and characterized, and used throughout the experiment. Routine phenotypic characterization showed isolate WK7 to be pigmented, circular with 1 mm colony diameter and entire margin while isolate WK12 appeared as non-pigmented, irregularly shaped with 1.5 mm colony diameter and entire margin when grown in a solid medium. Both isolates showed turbid and diffuse type of growth pattern in a liquid medium. Isolates WK7 and WK12 were further characterized to be aerobic, straight rod-shaped, motile, non-spore forming, catalase positive, and Gram negative. The isolates grew optimally in a medium containing 2% NaCl – their inability to grow in a medium not supplemented with NaCl indicated that they require saline conditions. Their cardinal growth temperatures were 25ºC to 37ºC – optimum at 30ºC. The isolates also presented growth at a

pH range of 6-10. Lastly, they were able to utilize sucrose and glucose and unable to utilize citrate and lactose as energy sources.

For the phylogenetic reconstruction, a total of 13 nucleotide sequences was used, this included the two (2) isolates used in this study, 10 related organisms, and one (1) outgroup. The two isolates that were used throughout the experiment (WK7 and WK12) showed high rDNA sequence similarities, close relationship, and consistent physiognomies to Shewanella and Pseudoalteromonas.

Two distinct clades were observed from the generated phylogenetic tree (Fig. 1). The first clade included isolate WK12 grouping with the genus Pseudoalteromonas. Isolate WK12 branched out from Pseudoalteromonas luteoviolacea NCIMB 1893 and P. byunsanensis FR1199, and showed a close relationship to P. prydzensis. It has been reported that closely related Pseudoalteromonas species such as P. byunsanensis and P. marina can be isolated from the tidal flat sediments of Korea. They are also common in the seas of Japan and Coral Sea of South China[9,10]. The Pseudoalteromonas species are known to be associated with marine invertebrates and are capable of producing highly brominated antimicrobial metabolites as exhibited by pentabromopseudilin from P. luteoviolacea[11,12]. Pigmented P. luteoviolacea has also been reported to produce a second metabolite compound that is a novel mono-brominated indole but it was not mentioned if the enzyme involved in producing the brominated metabolite is a haloperoxidase[12,13].

The second clade showed isolate WK7 grouping with genus Shewanella, the isolate being closely related to S. marisflavi. Shewanella species are frequently isolated in Yellow Sea, Korea[14,15]. Other related species (i.e. S. profunda and S. xiamenensis) can also be isolated from coastal seas and marine sediments[16,17]. In contrast to Pseudoalteromonas, there have been no reports yet on Shewanella producing brominated compounds.

BLAST analysis of the 16S rRNA gene sequences of the isolates confirmed that WK7 and WK12 were 99% identical to parts of the 16S rRNA gene of S. marisflavi strain SW-117 and P. prydzensis strain CM5, respectively. This is the first time that these isolates were reported to be capable of biocatalyzing

bromination reactions in MCD assay as resting whole cells. The sequences of isolates WK7 and WK12 were deposited to GenBank with the following accession numbers KP893637 and KP893638.

Analysis of biotransformation mixtures

The isolates were further tested for their bromination capability using indole as a substrate. The chromatogram of the standard substrate control for indole showed a peak eluted at t=24.82. When indole was incubated with the resting cells of P. prydzensis WK12 (KP893638), biotransformed products M1, M2, and M3 were observed; whereas products M4, M5, and M6 were detected in S. marisflavi WK7 (KP893637) incubated with indole.

Analysis of mixtures incubated with P. prydzensis WK12 (KP893638) or S. marisflavi WK7 (KP893637) and indole produced products M1 and M4 with $[M+H]^+$m/z 148.49 and 144.07 were both presumed to be isatin whose theoretical molecular mass is 147.13 g $(mol)^{-1}$. Isatin is an intermediate and derivative compound produced from indole when degraded, specifically when oxindole – one of the main products that can be obtained during bromination of indole – is hydroxylated [18].

Products M2 from P. prydzensis WK12 (KP893638) and M5 from S. marisflavi WK7 (KP893637) with the molecular ion peaks m/z 262.06 and 262.24 were speculated to be dimerization of indole containing one oxygen and additional hydrogen atom which can be achieved through monooxygenation of the parent compound. The compound was posited to be indigo as it bears the same mass with the theoretical mass of indigo which is 262.27 g $(mol)^{-1}$. The biotransformation of indigo from indole via oxidation pathways has been widely studied. As a result of the presence of high-yield biotransformed (possibly oxidation derived) products M2 and M5, an oxidation reaction was performed to verify if both isolates were capable of oxidation. An experiment was done by administering toluene as vapor in the resting cells of P. prydzensis WK12 (KP893638) and S. marisflavi WK7 (KP893637). Analysis of results showed presence of oxidized products observed at t=5.85 (M7) for P. prydzensis WK12 (KP893638), and t=5.83 (M8) and t=11.03 (M9) for S. marisflavi WK7 (KP893637). The m/z of M7 and M8 were 108.98 and $[M+H]^+$ 109.95, correspond to

the theoretical molecular mass of cresol which is 108.14 g $(mol)^{-1}$. The molecular ion peaks of M7 and M8 indicated the addition of an oxygen atom to the parent compound (toluene) which can only be achieved through oxidation reaction. The peak indicated as M9 has an m/z of 97.91. Based from the chromatogram of the extracted reaction mixtures, both isolate products (M7 from P. prydzensis WK12 KP893638 and M8 from S. marisflavi WK7 KP893637) showed the presence of an oxidized product of toluene which is cresol. It has been reported that chloroperoxidases and bromoperoxidases (halogenating enzymes) are also capable of oxidation reaction. Chloroperoxidase, in fact, can oxidize toluene into benzaldehyde and benzoic acid. It is possible then that the enzyme responsible for the oxidation of toluene is not a monooxygenase instead a bromoperoxidase[19,20].

Product M3 produced by the P. prydzensis WK12 (KP893638) isolate showed an $[M+H]^{+}$m/z of 342.03 indicating the addition of one bromine atom into the substrate M2. Bromine contains two isotopes namely ^{79}Br and ^{81}Br in a 1:1 ratio. In a mass spectral data, a compound having one bromine atom will exhibit two peaks with M+ and M+2m/z. It is expected then that any compound with bromine will show two identical peaks with a gap of 2 units of m/z in its spectrum. From the mass spectral data of product M3, this isotopic signature was observed, thereby substantiating that the compound it represents contains bromine, though in an unknown position. Product M3 matched the theoretical mass of a monobromoindigotin (MBI) which is 341.82 g $(mol)^{-1}$.

Lastly, S. marisflavi WK7 (KP893637) also produced a compound (M6) with m/z 420.57. Product M6 was assumed to be dibromoindigo – an analogue of the main component of Tyrian purple dye – based on its similarity to its molecular mass which is 420.05 g $(mol)^{-1}$. Due to bromine's spectral signature, an isotopic cluster comprising of three peaks in M+, M+2, and M+4m/z should be observed in the spectra of compounds having two molecules of bromine. Unlike compounds with one bromine, the peaks would have a high M+2m/z (middle peak), and nearly similar intensity/height M+ (first peak) and M+4m/z (last peak) – which are half of M+2m/z peak's intensity. Based on the spectrum of M6, this isotopic cluster was observed and in this manner confirms the presence of two bromine in the compound.

Conclusions

Two marine sediment bacteria with 99.9% similarity to parts of the 16S rDNA of S. marisflavi and P. prydzensis were found to be capable of transforming indole into indigo and subsequently biocatalyzing the bromination of indigo. Based on the mass spectral data of the reaction mixtures from isolates P. prydzensis (KP893638) and S. marisflavi (KP893637), possible monobromoidigo (m/z 342.03) and dibromoindigo (m/z 262.4) were produced. Few studies and none of which made use of whole resting cells of P. prydzensis and S. marisflavi, have successfully produced brominated and/or biotransformed products. The use of biocatalysts is now given more attention as most organic chemists prefer their favorable catalytic feature and exquisite selectivity. Industrial applications of microbes for the production of indole derived halogenated products have never been reported. This study ascertains that they can be further studied and developed to become industrially feasible, more so with the development of the modern biotechnology in which microbe-derived enzymes can now be prepared, and optimized as biotechnological tools for manufacturing purposes. Even though there have been no upfront preparative syntheses, it is an essential start that products have been detected analytically by research studies so that further developments can be done.

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Figure

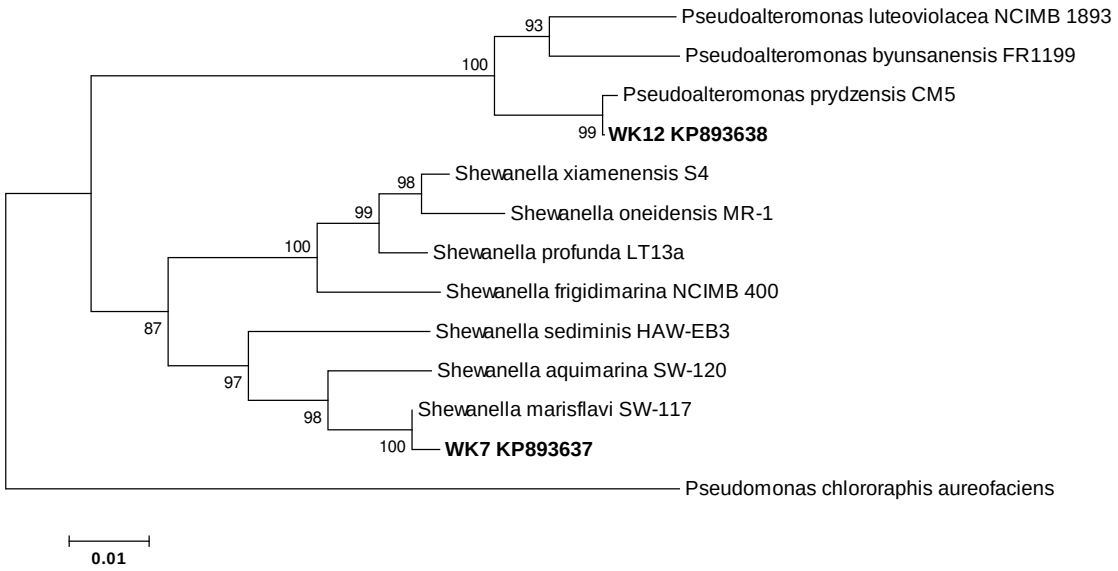

Fig. 1 Evolutionary relationships of isolates and other related organisms based on their 16S rDNA sequences. The isolates used in this study are labeled in bold letters, namely WK7 (KP893637) and WK12 (KP893638). The tree was inferred using Neighbor-Joining method with the sum of branch length = 0.34383801. The numbers beside the branches are bootstrap method percentages in which the associated taxa clustered together after 500 replications. The scale bar indicates 0.01 unit of number of base substitutions per site. Pseudomonas chlororaphis aureofaciens was included as an outgroup.