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Research article

Biodegradation of sulfamethoxazole in bacteria from three different origins

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ABSTRACT

Sulfamethoxazole (SMX) is a common medicine prescribed to treat infections. Due to vast use, SMX has been detected in different parts of the world. Hence, it has become a high risk because of its long term persistence with high biological activity in the ecosystem. Therefore, it is necessary to understand the mechanism of SMX degradation in different genus of bacteria, which is presently unclear. In the present study, degradation of 5 mg L^{-1} SMX was studied in three isolated pure bacterial cultures, *Ochrobactrum* sp. SMX-PM1-SA1, *Labrys* sp. SMX-W1-SC11 and *Gordonia* sp. SMX-W2-SCD14 and results showed up to 45.2%, 62.2% and 51.4% degradation, respectively within 288 h. Additionally, strain SA1 and strain SCD14 showed up to 66.2% and 69.2% of 4-aminophenol degradation at an initial concentration of 5 mg L^{-1} within 216 h whereas *Labrys* sp. SMX-W1-SC11 completely degraded 4-aminophenol at the same concentration within 120 h. Moreover, all three pure bacteria also completely degraded 3-amino-5-methylisoxazole at initial concentration of 4 mg L^{-1} within 120 h. Furthermore, gas chromatography-mass spectrometry and quadrupole time-of-flight mass spectrometry analysis results revealed that 3-amino-5-methylisoxazole, 4-aminophenol and hydroquinone were the three main by-products of SMX catabolism. In addition, cell free extracts of both *Labrys* sp. SMX-W1-SC11 and *Gordonia* sp. SMX-W2-SCD14 showed hydroquinone dioxygenase activity. Besides, all three bacterial strains showed resistance to different heavy metals. Moreover, all three pure bacterial cultures also showed positive chemotactic response toward 3-amino-5-methylisoxazole and hydroquinone based on the drop plate assay. The results of this study recommend these microorganisms for bioremediation of SMX contaminated sites.

1. Introduction

Sulfamethoxazole [4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide; SMX] is a commonly used medicine used to cure venereal infections (uncomplicated gonorrhea and chlamydia), gastrointestinal infections, central nervous system infections, respiratory infections and genitourinary tract infections (Kielhofner, 2005). Additionally, SMX is also used as herbicide in agriculture and aquaculture system (Boreen et al., 2004). Because of continuous usage, SMX has been observed in most part of world. For example, SMX was detected up to 113 ng L^{-1} in drinking water supply wells, 38–450 ng L^{-1} in ground water, 7.9–1900 ng L^{-1} in surface water (Lv et al., 2014; Wang et al., 2015) and up to 95.2 ng L^{-1} in the wastewater (Sun et al., 2016). Its long-term persistence in the environment might pose a high risk to the aquatic and neighboring living systems (Akhtar et al., 2011) and possibilities to enhance antibiotic resistant bacteria in the surrounding field (Ricken et al., 2015). Even at a lower concentration, it can induce genetic mutations and chronic effects (Zhang et al.,

Abbreviations: SMX, sulfamethoxazole; W1, wastewater; W2, activated sludge of a wastewater treatment plant; PM1, pig manure; AMS, ammonium mineral salts; MeOH, methanol; NCBI, national center for biotechnology information; ESI, electrospray ionization; BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; Q/TOF-MS, quadrupole time-of-flight mass spectrometry; MIC, minimum inhibitory concentration; Cu, Copper; Cd, Cadmium; Cr, Chromium; Co, Cobalt; Pb, Lead; Ni, Nickel; Zn, Zinc.

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2010) and therefore, it is necessary to remove and/or detoxify SMX from the contaminated sites.

Previous studies revealed that the chemical treatment methods like ozonation and electro-Fenton treatment generally cause the creation of toxic intermediates (Dantas et al., 2008; Dirany et al., 2011; Yargeau et al., 2008). Advanced oxidation and adsorption by activated carbon or membrane reactors can eliminate micropollutants including SMX from water (Dirany et al., 2011; Michael et al., 2013; Trovo et al., 2009). However, advanced oxidation and adsorption processes are costly, and hence, not fit in certain urban WWTPs. On the other hand, conventional techniques were used to remove micropollutants; however, certain micropollutants could not be completely removed from wastewater treatment plants (WWTPs), which represent serious challenge to the researchers.

Biological remediation is therefore expected to be an eco-friendly and cost-effective method for SMX removal from contaminated sites especially from wastewater. Till date, removal of SMX in pure bacteria, mixed cultures as well as sulfate reducing bacteria under aerobic conditions or anaerobic conditions was reported by researchers (Jia et al., 2017; Jiang et al., 2014; Kassotaki et al., 2016; Larcher and Yargeau, 2011; Muller et al., 2013; Reis et al., 2014; Ricken et al., 2015; Wang et al., 2015; Xu et al., 2011). However, degradation mechanism of SMX and its main by-products in different pure bacteria is still unclear. Hence, it is essential to isolate and identify different genus of potential pure bacteria having the ability to degrade SMX and its catabolic by-products simultaneously in the environment and also to characterize their ability to resist various heavy metals as well as their chemotaxic behavior towards SMX by-products. Generally, microbes have certain kinds of mechanisms which help them to adjust their cellular functional properties in response towards variations in its environmental system. Chemotaxis is a type of property present in the microbial cell which helps microorganisms to adjust their migration property under the influence of a substance gradient (Pandey and Jain, 2002). For example, the microorganism moves towards chemical substrate(s), which implies positive chemotaxis. Whereas the microorganism moves away from the substrate(s), the process is considered as negative chemotaxis (Arora et al., 2015; Pandey and Jain, 2002).

In this study, we isolated three different genera of pure bacteria, *Ochrobactrum* sp. SMX-PM1-SA1 (henceforth referenced as strain SA1), *Labrys* sp. SMX-W1-SC11 (henceforth referenced as strain SC11) and *Gordonia* sp. SMX-W2-SCD14 (henceforth referenced as strain SCD14) from different sources by enrichment technique on SMX. All three isolated pure bacterial strains showed the ability to degrade SMX and also its transformation by-products such as 4-aminophenol as well as 3-amino-5-methylisoxazole. The degradation pathway of SMX in all three bacteria were compared by GC-MS as well as Q/TOF-MS analysis and also by hydroquinone enzymatic study. Moreover, bacterial resistivity against different heavy metals were studied and reported here. Finally, chemotaxis of all three bacterial strains towards 3-amino-5-methylisoxazole and hydroquinone was also assessed by drop plate method. The results of this study might be helpful to enhance our understanding of SMX biodegradation.

2. Materials and methods

2.1. Chemicals and culture media

SMX was purchased from Sigma-Aldrich Co., USA. Acetone, acetonitrile and methanol were purchased from Merck, Germany. All other chemicals were of pure analytical grade and available commercially. Stock solutions of SMX, 4-aminophenol as well as 3-amino-5-methylisoxazole at 1 g L^{-1} were prepared in methanol and stored in amber bottles at −20 °C before use. The ammonium mineral salts (AMS) medium supplemented with yeast extract (0.04%) was prepared by method described previously (Mulla et al., 2016a) and the medium was set to pH 7.00 (using 2M NaOH or 2M HCl). The AMS medium was then distributed in 100 mL quantities into 250 mL Erlenmeyer flasks and sterilized by autoclaving for 20 min at 15 psi. For degradation study, specific substrates like SMX (5 mg L^{-1}), 4-aminophenol (5 mg L^{-1}) and 3-amino-5-methylisoxazole (4 mg L^{-1}) were added to the autoclaved AMS medium just before inoculation. Solid media contained 1.7–1.8% agar in AMS.

2.2. Enrichment of cultures and isolation of organisms

For enrichment technique, samples like wastewater (W1) and activated sludge (W2) of WWTPs (Mulla et al., 2016b) were collected from Xiamen whereas pig manure (PM1) was collected from Gaozhou District, Maoming City, China. The collected samples were used for the isolation of indigenous bacterial cultures by enrichment with SMX (6 mg L^{-1}) as a sole source of carbon and energy as described previously (Mulla et al., 2016b). In brief, 5 g and/or 5 mL of samples were dispensed in 100 mL of sterile milliQ water, mixed and filtered. The 5 mL of filtrates were transferred into 95 mL sterile AMS medium in 250 mL Erlenmeyer flasks supplemented with SMX. The enrichment cultures were then incubated aerobically under dark condition at 30 °C on a rotary shaker at 150 rpm. After one month, 5 mL of the inocula were transferred into fresh medium supplemented with SMX (6 mg L^{-1}). The flasks were again incubated for one month (until turbid, $O.D_{600}$ between 0.50 and 0.70). After 5 transfers, good growth was observed. Finally, the bacterial cultures were purified by serial dilution between 10^{-4} to 10^{-6} using 0.85% of sodium chloride (Reis et al., 2014) and were used for spread plate method. Cultured SMX (6 mg L^{-1})-AMS agar plates were kept in incubator at 30 °C for 48–72 h. Different colonies obtained from mixed microbial cultures were further grown on AMS agar plates containing SMX (6 mg L^{-1}). This process was repeated for several times to get pure individual bacteria. Based on growth response on SMX-AMS agar plates, three isolates designated as strain SA1, strain SC11 and strain SCD14 were identified and then preserved for further studies.

2.3. 16S rRNA gene sequence analysis for the identification of bacteria

Genomic DNA of each isolate was extracted using a TIANamp Bacteria DNA kit (Tiangen Biotech, Beijing, China). The 16S rRNA gene was amplified by PCR using forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer 1492R (5′-GGTTACCTTTGTTACGACTT-3′). PCR amplification was performed with a GenePro Thermal Cycler (Bioer, Hercules, China) (Mulla et al., 2016b). The 16S rRNA gene sequences of closely related taxa were obtained from the EzBioCloud database (Yoon et al., 2017). Multiple sequence alignment and phylogenetic analysis were performed using MEGA v7.0 (Kumar et al., 2016). Phylogenetic trees were constructed using the maximum-likelihood algorithm (Felsenstein, 1981) and tree topologies were evaluated using the bootstrap analysis of 500 replications.

2.4. Bacterial growth and degradation of SMX, 4-aminophenol as well as 3-amino-5-methylisoxazole

To assess the effect of various concentrations of SMX, 4-aminophenol and 3-amino-5-methylisoxazole on the growth of *Ochrobactrum* sp. SMX-PM1-SA1, *Labrys* sp. SMX-W1-SC11 as well as *Gordonia* sp. SMX-W2-SCD14 and their degradation, organisms