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Biodegradation of benzalkonium chlorides singly and in mixtures by a *Pseudomonas* sp. isolated from returned activated sludge

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HIGHLIGHTS

- *Pseudomonas* sp. degraded two benzalkonium chlorides: BDDA and BDTA.
- Although BDTA biodegraded at low concentration, it inhibited the degradation of BDDA.
- For BDDA, two transformation products indicate two sites of bacterial activity.
- ^{14}C-labelled BDDA was mineralized to $^{14}CO_2$ within 300 h.

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ABSTRACT

Bactericidal cationic surfactants such as quaternary ammonium compounds (QACs) are widely detected in the environment, and found at mg kg^{-1} concentrations in biosolids. Although individual QACs are amenable to biodegradation, it is possible that persistence is increased for mixtures of QACs with varying structure. The present study evaluated the biodegradation of benzyl dimethyl dodecyl ammonium chloride (BDDA) singly and in the presence of benzyl dimethyl tetradecyl ammonium chloride (BDTA) using *Pseudomonas* sp., isolated from returned activated sludge. Growth was evaluated, as was biodegradation using ^{14}C and HPLC-MS methods. BDTA was more toxic to growth of *Pseudomonas* sp. compared to BDDA, and BDTA inhibited BDDA biodegradation. The benzyl ring of [U-^{14}*C*-benzyl] BDDA was readily and completely mineralized. The detection of the transformation products benzyl methyl amine and dodecyl dimethyl amine in spent culture liquid was consistent with literature. Overall, this study demonstrates the antagonistic effect of interactions on biodegradation of two widely used QACs suggesting further investigation on the degradation of mixture of QACs in wastewater effluents and biosolids.

1. Introduction

Quaternary ammonium compounds (QACs), with long, hydrophobic alkyl chains attached to a positively charged nitrogen atom, are widely used in domestic, agricultural, healthcare, and industrial applications as surfactants, emulsifiers, fabric softeners, disinfectants, personal care products, and corrosion inhibitors [1]. The cationic character of QACs makes them highly sorptive to the negatively charged surfaces of sediments, minerals, and organic matter during the wastewater treatment process [2,3]. QACs can therefore enter the aquatic environment via the suspended organic matter in wastewater effluent [4], and the terrestrial environment through the application of biosolids as a soil amendment for crop production [5]. Total concentrations ranging from 22 to 167 mg kg^{-1} have been measured for QACs in biosolids [4,6]. Due to their extensive use, concentrations up to 3.8 μg L^{-1} have been found for QACs in surface water [7].

Benzalkonium chloride (BAC) compounds are a subset of QACs consisting of alkyl benzyl dimethyl ammonium chlorides with C_8–C_{18} alkyl groups [8]. The most widely used BACs have a carbon chain length of C_{12} or C_{14} and are found in concentrations up to 9 mg kg^{-1} dry weight in sewage sludge [6]. Grabińska-Sota [7]

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found potential genotoxicity in *Bacillus subtilis* in response to about 10 mg L^{-1} of a type of BAC. However, BACs were found to be genotoxic in eukaryotic cells even at a low concentration of 1.0 mg L^{-1} and their release into the environment may cause genetic damage in exposed organisms [9]. There is a concern that development of bacterial resistance to QACs will be accompanied by resistance to clinically relevant antibiotics [10]. Genetic elements that are associated with horizontal gene transfer of antibiotic resistance genes are enriched in QAC-polluted environments, and there is therefore a concern that exposure to QAC will accelerate the dissemination of antibiotic resistance within populations of environmental bacteria [11,12]. It should be noted, however, that environmental surveys have to date failed to establish a link between the use of QACs and the abundance of antibiotic resistant bacteria [1].

Biodegradation is the dominant process for BAC dissipation in the environment and therefore a crucial mechanism for reducing exposure and impacts on sensitive species [13]. Several studies concerning the biodegradation of QACs have been published [14–22]. Microorganisms capable of degrading QACs mostly belong to the genus *Pseudomonas* [14,16,18,20,22–26]. Some other species can also degrade QACs, including *Xanthomonas* sp. [22], *Bacillus niabensis*, *Thalassospira* sp. [15] and *Aeromonas* sp. [17]. However, while BACs are often found as mixtures in natural environments, the ability of microbes such as *Pseudomonas* to degrade mixtures of BACs and the synergistic or inhibitory effects of such mixtures on aerobic biodegradation have not been reported yet.

In the present study, a *Pseudomonas* sp., designated strain C505, was isolated from returned activated sludge (RAS) on the basis of its ability to use benzyl dimethyl dodecyl ammonium chloride (BDDA) as the sole source of carbon and nitrogen. The kinetics of biodegradation of BDDA and benzyl dimethyl tetradecyl ammonium chloride (BDTA) were established with each QAC provided alone or in a mixture. Some of the biodegradation intermediates and the extent of mineralization were also determined.

2. Materials and methods

2.1. Chemicals

Certified reference materials benzyl dimethyl dodecyl ammonium chloride (BDDA) and benzyl dimethyl tetradecyl ammonium chloride (BDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA); each had purity ≥ 99.0%. Radiolabeled BDDA (MC 2369), [benzyl-^{14}C; radioactive purity 98%, specific activity 50 μCi mL $^{-1}$ in ethanol or 52 mCi $mmol^{-1}$] was purchased from Moravek Biochemicals, USA and stored at −20 °C until use. Milli-Q water was obtained from an ultrapure water purification system (Millex-GV, 0.22 μm, Millipore, Toronto, ON, Canada). High performance liquid chromatography (HPLC) grade acetonitrile (purity ≥ 99.9%) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals used were of analytical reagent grade.

2.2. Maintenance and tentative identification of the bacterium

Strain C505, isolated from RAS collected from the Adelaide Pollution Control Plant in London, ON, Canada, was enriched in a minimal salts medium with BDDA provided as the sole carbon and nitrogen source.

The minimal salts medium [27] consisted of the following (per liter): 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.025 g $CaCl_2 \cdot 2H_2O$, and 1 mL of a trace metal solution consisting of the following: 10 mg $ZnSO_4.7H_2O$, 3 mg $MnCl_2.4H_2O$, 30 mg H_3BO_3, 20 mg $CoCl_2 \cdot 6H_2O$, 1 mg $CuCl_2 \cdot 2H_2O$, 2 mg $NiCl_2 \cdot H_2O$, 3 mg $NaMoO_4 \cdot 2H_2O$. The pH of the medium was 6.8 ± 0.2. The minimal salts medium was autoclaved at 120 °C for 20 min then supplemented with BDDA at 100 mg L^{-1} and 1 mL of freshly prepared 5 mg L^{-1} $FeSO_4.7H_2O$, which was sterilized using a 0.22 μm cellulose acetate filter (SteriCup, Millipore, Mississauga, ON, Canada) [28]. Liquid stock cultures of the isolated strain C505 were stored at 4 °C in minimal salts medium supplemented with 100 mg L^{-1} of BDDA. Before use, the strain was streaked on solid minimal salts medium containing 100 mg L^{-1} BDDA and 16 g L^{-1} agar and incubated for one week at 30 °C.

Strain C505 was presumptively identified as a *Pseudomonas* sp. on the basis of its 16S rRNA sequence. Genomic DNA was extracted from pelleted cells using the Promega Wizard kit (Thermo Fisher Scientific, Ottawa, ON, Canada) following the manufacturer's instructions. A partial 16S rRNA gene product was generated by PCR using primers that amplified a portion of the 16S rRNA gene GM5F (5′-CCTACGGGAGGCAGCAG-3′) [29] and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) [30]. The full length 16S rRNA product was generated using the primers FD1 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGHTACCTTGTTACGACTT-3′) [31].

The PCR conditions were 94 °C for 10 min, followed by 30 cycles of 94 °C for 20 sec, 55 °C for 30 sec and 72 °C for 45 s, with a final elongation step at 72 °C for 5 min. The PCR products were visualized on 1.5% agarose gel stained with GelRed to check for the expected sizes of 600 and 1500 base pairs using Molecular Imager, Gel Doc™XR+, with Image Lab™ software. The PCR products were purified using the QIAquick Gel extraction kit (Qiagen, Mississauga, ON, Canada) [28]. Sequencing was carried out at the Robart's Institute DNA Sequencing Facility at the University of Western Ontario by using a 3130xl Genetic Analyzer. The sequences were analyzed using BioEdit software; forward and reverse sequences were aligned to obtain the whole sequence and compared with nucleotide sequences in the GenBank database using BLASTn [32]. The sequence was within 99% similarity of several *Pseudomonas* species, most closely *Ps. putida* with 99.43% identity. On this basis and without further information the strain is designated as *Pseudomonas* sp. strain C505 (see supplementary materials Table S1 for details).

2.3. Analytical methods

Growth of the strain C505 was determined by measuring the optical density of the culture at 600 nm (OD_{600}) [18] using an Eppendorf Biophotometer (VWR International, Mississauga, ON, Canada). The relationship between OD_{600} and dry biomass was established for the strain C505 and is presented in the supplementary materials as Fig. S1. The OD_{600} was measured for each experimental sample, and the kinetic data were normalized by dry biomass to calculate the specific BACs degradation rate values [8].

Ultraviolet absorbance spectra of the culture supernatants were determined using a PowerWave XS microplate reader and Gen5 microplate reader software (Biotek, Winooski, VT, USA). Quantification of BACs was carried out using a high performance liquid chromatography (HPLC) coupled with a diode array UV-visible detector (Agilent Technologies, 1260 infinity, Mississauga, ON, Canada), equipped with a Luna CN column (C-18, 3 μm pore, 100 mm length × 4.6 mm internal diameter) with security guard CN column (Phenomenex, Canada) and the absorbance was measured at 210 nm [2]. The mobile phase consisted of acetonitrile and 40 mM ammonium acetate (adjusted to pH 5.0 using 1 M H_3PO_4) at a ratio of 60:40. The solvent gradient utilized was acetonitrile and 40 mM ammonium acetate for 0 to 20 min; 100% acetonitrile between 20 to 24 min; and acetonitrile: 40 mM ammonium acetate between 24 to 25 min, with a flow rate of 0.8 mL min^{-1} and an injection volume of 50 μL. The retention times for BDDA and BDTA were 11.9±0.2 min and 13.1 ± 0.2 min, respectively. Calibration curves