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Biohydroxylation of *N*,*N*-dialkylarylamines by the isolated topsoil bacterium *Bacillus megaterium*

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Abstract

Topsoil microorganisms were screened for their acceptability of the standard substrate *N*,*N*-dimethylaniline in bacterial 'whole-cell' incubations. One bacterium converted *N*,*N*-dimethylaniline and was identified as *Bacillus megaterium* by 16S rDNA analysis and DNA/DNA-hybridization. In contrast to the well-known *C*-hydroxylation by liver microsomes, leading to *p*-hydroxylation, *B. megaterium* formed *o*- and *p*-monohydroxylated products, i.e. *N*,*N*-dimethyl-2-aminophenol and *N*,*N*-dimethyl-4-aminophenol, both identified by gas chromatography–mass spectrometry (GC–MS) using synthesized reference compounds. The observed hydroxylation showed slight regioselectivity in favour of the *o*-hydroxylated product. Two further substrates, *N*,*N*-diethylaniline and *N*-ethyl-*N*-methylaniline, were also successfully biohydroxylated by *B. megaterium* with corresponding regioselectivity. Interestingly, aniline, known to be transformed easily by cytochrome P-450meg into *p*-aminophenol, was not accepted as substrate.

Keywords: *Bacillus megaterium*; Biohydroxylation; *N*,*N*-Dialkylarylamines; *N*,*N*-Dialkyl-2-aminophenols; *N*,*N*-Dialkyl-4-aminophenols

1. Introduction

Metabolic studies using *N*,*N*-dialkylarylamines have been carried out extensively by in vitro and in vivo experiments. In vitro studies have mostly been performed using liver microsomal preparations. Incubations with *N*,*N*-dialkylarylamines and subsequent analysis revealed products belonging to the phase-I-reaction metabolites like *N*-oxidation and *N*-dealkylation. *C*-Hydroxylation at the aryl ring yielded in *N*,*N*-dialkyl-4-aminophenols, only small amounts of *N*,*N*-dialkyl-2-aminophenols were obtained [1–3]. The in vivo metabolism of *N*,*N*-dialkylarylamines has been studied using dogs and rabbits [4,5]. After subcutaneous dosage the analysis of the excreted urine also revealed *N*,*N*-dialkyl-4-aminophenols whereas *N*,*N*-dialkyl-2-aminophenols have been detected only in traces. These reactions are most likely catalyzed by P-450 enzymes, being responsible for many biotransformations catalyzing hydroxylation and *N*-dealkylation reactions [6]. Microbial hydroxylation of *N*,*N*-dialkylarylamines with the aim to obtain the corresponding *N*,*N*-dialkylaminophenols has not been reported to date. One publication describes the 4-hydroxylation of aniline as substrate using the microbial enzyme system P-450meg from *Bacillus megaterium* ATCC 13368 [7,8]. Continuing our previous biocatalytical studies [9], a screening for the isolation of topsoil microorganisms was established, which are able to hydroxylate the aryl ring of *N*,*N*-dialkylarylamines. The isolated strain with the desired functionality was identified as *B. megaterium*. Herein, we describe the results of the observed biohydroxylation.

2. Experimental

2.1. Chemicals

N,*N*-Dimethylaniline **1**, *N*,*N*-diethylaniline **2**, and aniline **4** were obtained from Merck (Darmstadt, Germany). *N*-Ethyl-*N*-methylaniline **3** was purchased from Sigma–Aldrich (Taufkirchen, Germany). Compounds **1**–**4** and substances used for syntheses and microbial medium were of highest purity. The organic solvents used for synthesis and extraction were freshly distilled before use.

2.2. Isolation of topsoil bacteria

One gram of topsoil (collected from garden soil in Karlstadt, Germany) was stirred in sterile water for 20 min and 100 μl of this solution was plated on

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Standard Methods Agar plates (Bio Mérieux), each containing 10 μl of *N*,*N*-dimethylaniline **1** and incubated at 30 °C. Nitrogen-adapted microorganisms were singularized and cultivated on Standard Methods Agar plates. In total, 83 unknown cultures were obtained and the isolated strains were used for substrate acceptability tests by incubation in liquid media.

2.3. Substrate acceptability test with the isolated strains and isolation of the potential metabolites

Liquid minimal medium (75 ml) was prepared according to Dworkin and Foster [10], 5 ml l^{-1} of a *Pseudomonas* trace element solution was added and the mixture was autoclaved (121 °C, 16 min) [11]. The culture was maintained under sterile conditions during the addition of 750 μl of a sterile glucose solution (50%). This liquid medium was inoculated with freshly grown bacteria and the culture was pregrown for 24 h at 30 °C and 120 rpm. Then 100 μmol of the used standard substrate *N*,*N*-dimethylaniline **1** (or substrates **2**–**4**) were added and shaken for further 24 h at 30 °C and 120 rpm. Control experiments without bacteria were carried out to verify the stability and authenticity of the starting material. After shaking, the culture was worked up by sonication of the bacterial broth for 10 min and centrifugation at 5,000 × *g* for 20 min. The supernatant was extracted four times each with 40 ml of diethyl ether. The combined organic layers were dried over Na_2SO_4, filtrated and the organic solvent was evaporated under reduced pressure. The residue was redissolved in 500 μl of dichloromethane. The products were submitted to gas chromatographic–mass spectrometric (GC–MS) analysis, and their identity was verified by comparison of their chromatographic and MS data with that of synthesized references prepared as described in Section 2.6.

2.4. Genotypic characterization of the isolated strain

2.4.1. RIDOM 16S rDNA sequencing of the isolated bacteria

DNA preparation, DNA amplification and DNA sequencing of the 16S ribosomal RNA gene were performed as previously described [12]. Briefly, the thermal cycling conditions consisted of an initial denaturation (80 °C, 5 min) followed by 28 cycles of denaturation (94 °C, 45 s), annealing (53 °C, 1 min), and extension (72 °C, 90 s), with a single final extension (72 °C, 10 min). For amplification, the broad-range primers 16S-27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 16S-907r (5′-CCG TCA ATT CMT TTR AGT TT-3′) reported by Lane were applied [13]. The PCR product was purified by an enzymatic method with exonuclease I (New England Biolabs GmbH, Frankfurt-Hoechst, Germany) and shrimp alkaline phosphatase (Amersham Biosciences, Freiburg, Germany) [14]. The amplicons were sequenced using the Prism BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). The sequencing reaction required 0.5 μl of premix from the kit, 1.8 μl Tris–HCl/$MgCl_2$ buffer (400 mM Tris–HCl; 10mM $MgCl_2$), 10 pmol of sequencing primer, and 2 μl of the cleaned PCR product in a total volume of 10 μl. For 16S rDNA sequencing either the primer 16S-27f or 16S-519r (5′-GWA TTA CCG CGG CKG CTG-3′) were used with an annealing temperature of 53 or 60 °C, respectively. All sequencing reactions were performed using a T1 thermocycler (Whatman Biometra, Göttingen, Germany) with 25 cycles of denaturation (96 °C, 10 s), annealing (5 s), extension (60 °C, 4 min) and a thermal ramping of 1 °C/s. The sequencing products were purified with MultiScreen HV plates (Millipore, Billerica, MA, USA) loaded with Sephadex G50 Superfine columns (Amersham Biosciences) according to the instructions of the manufacturer (Millipore Tech Note TN053); followed by preparation for running onto the ABI Prism 3100 Avant Genetic Analyser. The region from base positions 54 to 510 (corresponding to *Escherichia coli* 16S rDNA positions) for the 16S rDNA were analyzed using the Ridom TraceEdit Pro (Version 0.8 beta; Ridom GmbH, Würzburg, Germany) software. Sequences from primer regions were therefore not included in this analysis. Finally, a homology search of the sequence data against the RIDOM database was performed [15,16].

2.4.2. DNA/DNA-hybridization of the isolated B. megaterium against B. megaterium ATCC 14581 type strain (DSM 32^T)

The isolated strain, identified as *B. megaterium* by 16S rDNA analysis was also subjected to a DNA/DNA-hybridization analysis to reassess the correctness of the given species name. *B. megaterium* ATCC 14581 (DSM 32^T) type strain was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Deutschland) and cultivated in liquid media (Medium 1, DSMZ) with the same incubation conditions as described above. DNA/DNA-hybridization analysis was performed by DSMZ. Briefly, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite [17]. DNA/DNA-hybridization was carried out as described by De Ley et al. [18] under consideration of the modifications described by Huss et al. [19] using a model Cary Bio UV/Vis-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian).

2.5. Deposition of the isolated strain

The isolated *B. megaterium* strain was deposited in the BCCM/LMG, Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent (RUG), B-9000 Gent, Belgium, under the LMG no. 23147.

2.6. Synthesis of hydroxylated N,N-dialkylarylamines

The general procedure for the synthesis of *N*,*N*-dialkyl-2-aminophenols **1a**–**3a** has been described by Kalgutkar et al. [20]. The synthesis of *N*,*N*-dialkyl-4-aminophenols **1b**–**3b** was performed according to Lee et al. [21]. The synthesized products were submitted to GC–MS analysis (Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass selective detector; J&W DB Wax fused-silica capillary column: 30 m × 0.25 mm i.d., 0.25 μm film thickness) and nuclear magnetic resonance (NMR) spectroscopy (Bruker Avanced-400 spectrometer at 400 MHz) to verify their identity.

3. Results and discussion

In the screening process for microbial hydroxylation of *N*,*N*-dimethylaniline **1** one strain was found to be able to hydroxylate the offered substrate. This soil isolate was characterized by the standard 16S rDNA method and DNA/DNA-hybridization analysis. A direct comparison of the 16S rDNA partial sequences indicated that the isolate belongs to the species *B. megaterium* showing a similarity of 99.6% for 456 base pairs against the *B. megaterium* type strain (ATCC 14581, DSM 32^T). To complete the assignment of the isolate to the species *B. megaterium*, DNA/DNA-hybridization against the *B. megaterium* type strain was conducted. A mean DNA/DNA-similarity value of 83.2% was obtained. Therefore, the assignment of the isolate to *B. megaterium* was justified and the results of the 16S rDNA analysis were supported by this technique.

The isolated *B. megaterium* was found to hydroxylate the aryl ring of **1**. The hydroxylation capacity was proven with two further *N*,*N*-dialkylarylamines, i.e. *N*,*N*-diethylaniline **2** and *N*-ethyl-*N*-methylaniline **3** as substrates, resulting in the metabolites outlined in Table 1.

Both 2- and 4-hydroxylation occurred, with a slight preference of the 2-hydroxy conversion product. Among **1**–**3**, *N*,*N*-diethylaniline **2** was the preferred substrate as shown by the hydroxylation yields in Table 1. This finding is in contrast to former metabolic studies, where 4-hydroxylation of *N*,*N*-dialkylarylamines dominated and 2-hydroxlation was only a minor pathway [1–5]. Surprisingly, aniline **4** used as substrate was not accepted by the isolated *B. megaterium*, a result that disagrees with previous findings of Berg and Rafter [8] who have described the 4-hydroxylation of **4** using P-450meg from *B. megaterium* ATCC 13368. Thus, it can be suggested that P-