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Nocardioides carbamazepini sp. nov., an ibuprofen degrader isolated from a biofilm bacterial community enriched on carbamazepine

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ABSTRACT

From the metagenome of a carbamazepine amended selective enrichment culture the genome of a new to science bacterial species affiliating with the genus *Nocardioides* was reconstructed. From the same enrichment an aerobic actinobacterium, strain CBZ_1^T, sharing 99.4% whole-genome sequence similarity with the reconstructed *Nocardioides* sp. bin genome was isolated. On the basis of 16S rRNA gene sequence similarity the novel isolate affiliated to the genus *Nocardioides*, with the closest relatives *Nocardioides kongjuensis* DSM19082^T (98.4%), *Nocardioides daeguensis* JCM17460^T (98.4%) and *Nocardioides nitrophenolicus* DSM15529^T (98.2%). Using a polyphasic approach it was confirmed that the isolate CBZ_1^T represents a new phyletic lineage within the genus *Nocardioides*.

According to metagenomic, metatranscriptomic studies and metabolic analyses strain CZB_1^T was abundant in both carbamazepine and ibuprofen enrichments, and harbors biodegradative genes involved in the biodegradation of pharmaceutical compounds. Biodegradation studies supported that the new species was capable of ibuprofen biodegradation. After 7 weeks of incubation, in mineral salts solution supplemented with glucose (3 g l^{-1}) as co-substrate, 70% of ibuprofen was eliminated by strain CBZ_1^T at an initial conc. of 1.5 mg l^{-1}.

The phylogenetic, phenotypic and chemotaxonomic data supported the classification of strain CBZ_1^T to the genus *Nocardioides*, for which the name *Nocardioides carbamazepini* sp. nov. (CBZ_1^T = NCAIM B.0.2663 = LMG 32395) is proposed.

To the best of our knowledge, this is the first study that reports simultaneous genome reconstruction of a new to science bacterial species using metagenome binning and at the same time the isolation of the same novel bacterial species.

Introduction

An unprecedented increase in the consumption and simultaneous release and accumulation of pharmaceuticals in the environment can be witnessed worldwide due to the growing population. The review of Patel et al. [1] summarizes the occurrence of pharmaceutical residues in aquatic and terrestrial ecosystems, and it makes obvious that pharmaceutical residues are present in surface water, groundwater and even in drinking water.

The most commonly detected pharmaceuticals in the freshwater ecosystems are diclofenac (DIC), ibuprofen (IBU) and carbamazepine (CBZ), they can be detected in surface- and groundwater, as well as finished drinking water [2–4]. These compounds enter the environment mainly through municipal and industrial wastewater effluents [2].

Pharmaceutical residues are gaining more and more attention since they can produce adverse effects on aquatic organisms. It has been demonstrated that at environmentally relevant

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concentrations DIC, IBU and CBZ may exert ecotoxic effect on rainbow trout (*Oncorhynchus mykiss*), mussels (*Mytilus galloprovincialis* and *Dreissena polymorpha*), crustacean (*Gammarus pulex*), adult zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) among others [5–11]. Therefore, it must be a priority to understand the ultimate environmental fate of pharmaceutical residues and to investigate the microbiology behind their biodegradation.

We recently committed to the selective enrichment, identification and isolation of pharmaceuticals-, especially DIC, IBU and CBZ, degrading bacteria. In the present paper we report on a novel *Nocardioides* species capable of ibuprofen biodegradation obtained from a biofilm bacterial community selectively enriched on carbamazepine as sole source of carbon and energy.

Based on the List of Prokaryotic Names with Standing Nomenclature (www.bacterio.net; [12]), the genus *Nocardioides* currently comprises 136 validly described species. The genus was proposed by Prauser [13]. *Nocardioides* species are Gram-positive, coccus-shaped actinobacteria often isolated from diverse habitats such as seawater, freshwater spring, soil, sediments or even from xenobiotics contaminated environments [14–23,14,24]. Some members of the genus such as *N. aromaticivorans* H-1^T, *N. nitrophenolicus* NSP 41^T, *N. oleivorans* BAS3^T, *N. daeguensis* JCM 17460^T, *N. pyridinolyticus* OS4^T and *N. soli* mbc-2^T were described as dibenzofuran, p-nitrophenol, crude-oil, chlorophenols, pyridine and carbendazim degrading organism, respectively [19–21,25,26]. Other *Nocardiodes* isolates have been reported as atrazine, phenanthrene, s-triazine herbicide, deoxynivalenol and 2–4-dinitroanisole degrading bacteria [27–31]. To the best of our knowledge no *Nocardioides* pure isolates have been reported so far capable of ibuprofen biodegradation.

Materials and methods

Selective enrichment cultures

The selective enrichment of potentially pharmaceutical degrading bacteria from a microbial biofilm was conducted in mineral salts solution amended with vitamins [32]. The enrichment cultures contained as sole source of carbon and energy either diclofenac sodium, ibuprofen or carbamazepine (100 mg l^{-1}). The biofilm sample was collected from a Pump & Treat system treating BTEX (benzene, toluene, ethyl-benzene, xylenes) contaminated groundwater. Both the groundwater remediation system and the starting biofilm bacterial community were thoroughly characterized earlier. The biofilm bacterial community has already been proven to be the source of isolation of simple- and polycyclic aromatic hydrocarbon degrading bacteria [33–35].

The enrichments were done in 300 ml Erlenmeyer flasks (sealed with cotton wool) containing 100 ml of enrichment media inoculated with 1 ml of biofilm bacterial suspension (0.5 g biofilm suspended in 50 ml saline solution – 0.9% NaCl). The enrichment cultures were incubated at 28 °C on a rotary shaker (150 rpm) for 3 months in total. After one and two months of incubation sub-cultivations took place; 10 ml of the enrichment culture was transferred to 90 ml of fresh enrichment medium and incubated again for one additional month.

Metagenomic and metatranscriptomic studies of enrichment cultures

Genomic DNA and mRNA purification

To assess the phylogenetic diversity of the samples, shotgun metagenome sequencing was performed on the total community DNA of the initial biofilm sample and of the pharmaceutical amended enrichment cultures using Illumina platform (Illumina Inc., USA). Total community DNA from the initial biofilm sample (0.5 g) and the enrichment cultures of each month was extracted using the DNeasy® PowerBiofilm Kit (Qiagen, Germany) following the instructions of the manufacturer. From enrichment cultures, 40 ml were centrifuged at 2360 *g* for 15 min using a Rotanta 460 R centrifuge (Hettich, Germany) and the community DNA was extracted from the pellet.

The quantity of DNA samples was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and a Qubit 2.0 Fluorimeter (Life Technologies, Carlsbad, USA). Paired-end fragment libraries were prepared using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq® 500/550 High Output Kit v2 (300 cycles).

For the metatranscriptomic study, total RNA from the pellet (40 ml of enrichment culture centrifuged at 2360 g) of the three months old enrichment cultures was extracted using PureLink™ RNA Mini Kit (Invitrogen, Thermo Fisher Scientific). Metatranscriptome sequencing was performed as follows: paired-end libraries were prepared using the Zymo-Seq RiboFree™ Total RNA Library Kit (the protocol includes the universal rRNA depletion step as well). Sequencing was done on an Illumina NextSeq sequencer using the TG NextSeq® 500/550 High Output Kit v2 (300 cycles). Primary data analysis (base-calling) was carried out with "bcl2-fastq" software (v2.17.1.14, Illumina).

Raw sequence filtering and co-assembly

Galaxy Europe server was employed to pre-process the raw sequences (i.e., sequence filtering, mapping, quality checking). Low-quality reads were filtered by Prinseq (min. length: 100; min. score: 15; quality score threshold to trim positions: 20; sliding window used to calculated quality score: 1). Filtered sequences were checked with FastQC. The filtered sequences produced by Prinseq were co-assembled with Megahit [36] (min. contig length: 1500; min k-mer size: 21; max k-mer size: 141). Downstream taxonomical analysis and visualization of the retained metagenomic sequence reads were performed by the MEGAN6 software [37]. Raw metagenomic and metatranscriptomic sequence reads are available on NCBI under the following BioProject accession numbers PRJNA782474.

Reconstruction of bacterial genomes from the metagenomes (metagenome binning)

The quality filtered, trimmed and assembled reads obtained from the previous step were used for assembling genomes from the metagenomes. Binning of the contigs was carried out with three different binning algorithms: Metabat2 [38], Maxbin2 [39] and Concoct [40]. The result of each binning procedure was further improved with Metawrap [41]. Bin qualities were estimated with CheckM [42] and bin taxonomy was determined using the GTDB taxonomic database [43]. The metagenome assembled genome relevant to this study, *Nocardioides* sp. bin2, can be accessed through NCBI under the accession number JAJTIU000000000.

Calculation of RPKM values based on metagenome and metatranscriptome data

Phylogenetic diversity of metagenomes indicated that the relative abundance of *Nocardioides* spp. was considerable in CBZ and IBU enrichments. In connection with this, genome-resolved metagenomics resulted in a high quality *Nocardioides* related bin genome (100% completeness, NCBI accession number JAJTIU000000000).

To get information about the incidence and activity of the bin related *Nocardioides* species in different enrichment cultures during the whole enrichment period, RPKM (read per kilobase per million mapped reads) values were calculated using metagenome data obtained from enrichment cultures and the reconstructed bin

genome. In the case of the three-months-old enrichment cultures the RPKM values were calculated also on metatranscriptome data.

The RPKM values were calculated as follows:

$$RPKM = number\ of\ mapped\ reads / \left(\frac{genome\ length}{1000} * \frac{total\ number\ of\ reads}{1,000,000}\right)$$

where the "number of mapped reads" were measured by Bowtie2. First, Bowtie2 was used to create library from the *Nocardioides* sp. bin2 related bin genome, then it was mapped back to the whole gDNA sequences and mRNA sequences originated from the enrichment cultures. The "genome length" is the size of the used bin genome, and the "total number of reads" are the number of filtered reads produced by Prinseq.

Bacterial isolation and studied bacterial strains

The isolation of bacterial strains from the enrichment cultures was performed after each month of incubation; ten-fold serial dilutions in saline solutions were made and inoculated onto R2A agar plates (proteose peptone 0.5 g, casamino acids 0.5 g, yeast extract 0.5 g, dextrose 0.5 g, soluble starch 0.5 g, dipotassium phosphate 0.3 g, magnesium sulfate·$7H_2O$ 0.05 g, sodium pyruvate 0.3 g, agar 15 g, pH 7 ± 0.2). The plates were incubated at 28 °C for 48 h. The developed colonies were purified by streak plating and maintained on R2A agar slants at 4 °C, and stored long-term at −80 °C in a glycerol-R2A solution (30% v/v).

A bacterial strain forming whitish, translucent, flat colonies with irregular margins on R2A agar, the representative of the new bacterial species on which this study is based on, *Nocardioides* sp. nov. CBZ_1^T, was isolated from the one-month-old carbamazepine enrichment.

The reference organisms used for taxonomic studies were type strains of *Nocardioides* species such as *N. kongjuensis* DSM19082^T, *N. nitrophenolicus* DSM15529^T and *N. daeguensis* JCM17460^T. The strains with DSM and JCM numbers were obtained from the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and the Japan Collection of Microorganisms, RIKEN (Wako, Japan), respectively.

At different stages of the study, for the cultivation of all strains R2A medium was used, unless specified otherwise.

16S rRNA gene sequencing and phylogenetic analyses

Genomic DNA of isolate CBZ_1^T was extracted by using the DNeasy® UltraClean® Microbial DNA isolation Kit (Qiagen, Germany). Bacterial species specific 16S rRNA genes were PCR amplified by using the universal bacterial primers 27F (5′-AGAGTTTGATC(A/C)TG GCTCAG-3′) and 1492R (5′-TACGG(C/T)TACCTTGTTACGAC TT-3′). The PCR reaction mixture in a final volume of 50 µl contained, 5 µl 10 × DreamTaqTM buffer (ThermoFisher Scientific, Lithuania) with MgCl2 (2 mM), 0.2 mM of each dNTP, 0.1 µM of each primer, 1 U of DreamTaqTM DNA Polymerase (ThermoFisher Scientific, Lithuania), ∼30 ng extracted DNA and nuclease free water up to the final volume. The temperature profile used for the amplification was an initial annealing for 3 min at 95 °C followed by 32 cycles of denaturation for 30 sec at 94 °C, annealing for 30 s at 52 °C, elongation for 1 min at 72 °C and then a final extension for 10 min and 10 s at 72 °C. All amplifications were analyzed under UV light after electrophoresis in 1% (w/v) agarose gel stained with ethidium-bromide. The PCR amplicons were purified by using NucleoSpin® Gel and PCR Clean-up set (Macherey-Nagel, Germany). Subsequently, 16S rRNA gene nucleotide sequences were determined with Sanger-sequencing by using BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) the sequences were analyzed with ABI 3130 Genetic Analyzer (Life Technologies, USA). The resulted sequences were edited and assembled using MEGA X [44], then homology BLAST searches [45] were made in the GenBank database (https://www.ncbi.nlm.nih.gov/BLAST/). EzTaxon-e server carried out the determination of the closest type strains of isolates on the basis of 16S rRNA genes (https://eztaxon-e.ezbiocloud.net/, [46]. 16S rRNA gene sequence of strain CBZ_1^T was deposited in the GenBank under the accession number MZ408918.

Multiple alignment of sequences, calculation of evolutionary distance by Kimura's two parameter model [47], and construction of a neighbor-joining [48] phylogenetic tree were performed using MEGA X. The topology of the trees was evaluated by bootstrapping with 10,000 resamplings [49].

Phylogenomic tree reconstructions

As a first attempt, for genome-based phylogeny the UBCG (up-to-date bacterial core gene) phylogenomic pipeline and 92 core genes (UBCGs) were used. The phylogenetic tree was done for each gene as well as a concatenated sequence of the 92 UBCGs (UBCG tree) using the available genomes on the NCBI of those *Nocardioides* species that were used for the generation of the 16S rRNA gene based phylogenetic tree. The tree generated from the concatenated alignment is representing the true evolutionary history of whole genomes, however it may be different from those generated using individual gene trees. To estimate the robustness of each branch the Gene Support Index (GSI) was calculated. The higher the GSI the more robustly the branch is supported [50].

To confirm the obtained UBCG phylogenetic inference, a second phylogenomic tree was generated using 101 bacterial whole genomes and 120 bacterial marker genes belonging to type strains of different *Nocardioides* species. As a first step, the GTDB-Tk classify_wf workflow was used for the generation of GTDB-Tk MSA file that was used for the generation of the phylogenetic tree using the IQTree program (number of bootstrap alignments 1000, maximum iteration 1000, minimum correlation coefficient 0.99, perturbation strength 0.5, IQTree stopping rule 100). Subsequently, the obtained Newick file was visualized with the iTOL program.

Pan-genomic study and the environmental relevance of the novel Nocardioides sp.

To see the difference in genomic content between the novel species and other *Nocardioides* spp. pan-genome analysis was performed with the closest 23 *Nocardioides* spp. determined on the basis of the whole-genome based phylogenetic tree (Fig. S1, thickened branch).

Using Anvi'o 7 a database was created from the 25 genomes (anvi-gen-contigs-database and anvi-gen-genomes-storage). From the obtained database a pan-genome database was generated using anvi-pan-genome. The pan-genome database was supplemented with ANI calculations using the FastANI program within Anvi'o (anvi-compute-genome-similarity). The obtained results were visualized with anvi-display-pan and summarized using anvi-summarize. Unique genomic islands (UGIs) characteristic to the novel species were also determined.

In order to see if the obtained novel *Nocardioides* sp. is present in other metagenomes, e.g. those exposed to pharmaceutical compounds, recruitment of metagenomic reads against the genome of strain CBZ_1^T and the reconstructed *Nocardioides* sp. bin2 genome was done and the relative abundance values were calculated using the CoverM pipeline (https://github.com/wwood/CoverM). For this step metagenomic sequence reads from pharmaceutical (trimethoprim, sulfamethoxazole and carbamazepine) amended liquid enrichment cultures were used from a previous study ([51]; BioProjectAccession PRJNA286671).

Morphological, physiological, and biochemical tests

Cell size, shape and arrangement of the type strain CBZ_1^T were studied by native preparations and by Gram staining according to Claus [52]. The cell morphology was investigated using transmission electron microscopy (Morgagni 268). For transmission electron microscopy cells were negatively stained with 1% (w/v) uranyl acetate [53]. The growth of strain CBZ_1^T in different growth media such as R2A, nutrient (proteose peptone 5 g, beef extract 3 g, NaCl 5 g), LB (tryptone 10 g, yeast extract 5 g, NaCl 5 g) and TSA – trypticase soy (tryptone 15 g, soy peptone 5 g, NaCl 5 g) was determined. The temperature tolerance of the isolate was assessed in R2A medium at 4, 15, 23, 28 and 37 °C. The optimum pH for growth was assessed in R2A broth and the medium was adjusted to pH 4.0–12.0 (at intervals of 1 pH unit) using citrate/NaH_2PO_4 buffer (0.1 M, for pH range 4.0–5.0), phosphate buffer (0.1 M, for pH range 6.0–7.0), Tris buffer (0.2 M, for pH range 8.0–10.0) and NaOH (5 M, for pH range 10.0–12.0). Tolerance towards salinity was determined by inoculating the strain into R2A broth supplemented with 0–4% (w/v) NaCl at 1% intervals. During determination of optimal cultivation conditions and tolerance tests the bacterial growth was followed by measuring the optical density (OD) of the bacterial suspension at 600 nm. Growth under anaerobic conditions was assessed in R2A broth with and without the addition of 0.15% (w/v) KNO_3 at 28 °C similar to Révész et al. [54]. To ensure anaerobic conditions 100 ml serum bottles containing 50 ml of R2A broth were hermetically closed and sparged with N_2/CO_2 (80:20, v/v) under sterile conditions. Dissolved oxygen concentration in the bottles was measured non-invasively by using a Fibox 3 trace version 3 fibre optic oxygen meter with PSt3 sensor spots (PreSens).

Physiological and biochemical tests such as production of hydrogen sulphide from cysteine, hydrolysis of Tween 80, gelatin, casein and starch, and DNase activity, were performed according to the protocols of Barrow and Feltham [55]. Catalase activity was determined by bubble production from H_2O_2 (3%, v/v) and oxidase activity was tested using 1% (w/v) tetramethyl-phenylenediamine oxalate.

API® 50CH, API® 20NE and API® ZYM strips (bioMérieux, France) were used to further determine biochemical characteristics of strains CBZ_1^T and the selected closest relatives following the instructions of the manufacturer.

Chemotaxonomic analyses

Chemotaxonomic analyses were conducted by the Leibniz Institute, DSMZ (Braunschweig, Germany).

Respiratory quinones were extracted from freeze dried cell material using hexane and were further purified by a silica-based solid phase extraction. Purified samples were further analyzed by HPLC using a reverse phase column recording absorption spectra. 270 nm for ubiquinones and 326 nm for menaquinones were used for a relative quantification.

Polar lipids were extracted from freeze dried biomass using a chloroform: methanol:0.3% aqueous NaCl mixture, and were recovered into the chloroform phase (modified after [56]). Polar lipids were separated by two-dimensional silica gel thin layer chromatography. The first direction was developed in chloroform:methanol:water, and the second in chloroform:methanol:acetic-acid:water. Total lipid material was detected using molybdatophosphoric acid [57].

The peptidoglycan structure of strain was determined from wet biomass (centrifugal pellet suspended in isopropanol/water 1:1, v/v) as described by Schumann [58].

For cellular fatty acid determinations all strains were cultivated on the same growth medium (R2A). Cellular fatty acids of strain CBZ_1^T and the closest relatives were analyzed after conversion into fatty acid methyl esters (FAMEs) by saponification, methylation and extraction using minor modifications of the method of Miller [59] and Kuykendall [60]. The fatty acid methyl esters mixtures were separated by gas chromatography and detected by a flame ionization detector using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.). Peaks were automatically integrated and fatty acid names and percentages calculated by the MIS Standard Software (Microbial ID).

Whole genome sequencing (WGS) and analysis

The genome of strain CBZ_1^T was sequenced as described earlier in Borsodi [61]. Briefly, Nextera Mate Pair Sample Preparation Kit (Illumina, U.S.A) was used to generate mate-paired libraries according to the manufacturer's protocol for gel-plus version with slight modifications. A total of 13 µl of Mate-Paired Tagment Enzyme was used to produce a robust smear within the 7–11 kbp region. The 7–11 kbp DNA fraction was excised from the gel using the Zymoclean Large Fragment DNA Recovery kit (Zymo Research, U.S.A) and the circularized DNA was sheared using Covaris S2. All quality measurements were performed on a TapeStation 2200 instrument (Agilent, U.S.A). Final libraries were quantified using Qubit (ThermoFisher, U.S.A) and sequenced on an Illumina MiSeq instrument using MiSeq Reagent Kit v2 (500 cycles) sequencing chemistry. *De novo* assembly and scaffolding were performed with CLC Genomics Workbench Tool v11 (Qiagen, Germany). The genome accession number of *Nocardioides* sp. nov. CBZ_1^T is: JAHTLF000000000.

To ascertain the precise taxonomic position of strain CBZ_1^T, using the available bacterial genomes, the ANI (orthologous average nucleotide identity) and dDDH (*in silico* digital DNA-DNA hybridization) values between strain CBZ_1^T and the closest relatives were calculated using Ez-Biocloud (https://www.ezbiocloud.net/tools/ani; [62]) and the server-based genome-to-genome distance calculator version 2.1 (https://ggdc.dsmz.de/ggdc.php#; [63]), respectively. ANI and dDDH values between strain CBZ_1^T and the reconstructed *Nocardioides* sp. bin2 were also determined.

No publicly accessible genome sequence of *N. daeguensis* JCM 17460^T was available. Consequently, for genome-based phylogenetic studies the genomic DNA of strain JCM 17460^T was extracted and sequenced during this study similar to WGS of strain CBZ_1^T. The genome accession number of *N. daeguensis* type strain JCM 17460^T is: JAHUBO000000000.

Annotation of the genome of strain CBZ_1^T was performed by the Microbial Genome Annotation & Analysis Platform MicroScope (MaGe) [64]. Additionally, putative functions of genes associated in the metabolism of xenobiotics were identified and bioinformatically analyzed by using MaGe in conjunction with the UniProt database (https://www.uniprot.org/; [65]) and BLAST searches.

Testing pharmaceutical biodegradation capacity of strain CBZ_1^T

The type strain CBZ_1^T was tested for its ability to degrade carbamazepine and ibuprofen. Initially, the biodegradation tests were conducted in triplicates in 50 ml of Bushnell-Haas mineral medium ($CaCl_2{\cdot}2H_2O$ 0.002 g, $MgSO_4{\cdot}7H_2O$ 0.02 g, NH_4NO_3 1 g, KH_2PO_4 1 g, K_2HPO_4 1 g, $FeCl_3{\cdot}6H_2O$ 0.005 g, H_2O 1 l, with pH 7) supplemented with one of the before mentioned pharmaceuticals as sole source of carbon and energy (1.5 mg l^{-1}). Subsequently, co-metabolic biodegradation tests were also conducted, easily assimilable carbon sources such as yeast extract (0.05 or 0.3 g l^{-1}), or glucose (0.5 or 3 g l^{-1}) were added to the test solutions mentioned above next to the pharmaceutical compounds.

The test solutions were inoculated with 50 µl of bacterial cell suspensions (OD_{600} = 1) obtained in physiological saline solution. Abiotic controls, containing all the above mentioned except the

bacterium were also set up. Test runs were incubated for several weeks on a rotary shaker at 145 rpm and 27 °C.

The concentration of pharmaceutical compounds in the bulk solution was determined weekly using high performance liquid chromatography (HPLC). Prior to injection into the HPLC instrument the aqueous samples were filtered by passing through Whatman cellulose acetate syringe filters (0.45 μm). A Chromaster Hitachi instrument consisting of a Model 5110 pump, a Model 5210 autosampler, and a Model 5430 Diode-array detector (DAD) was used. The separation and data processing were operated by a EZChom Alite software. The separation of the pharmaceutical compounds was performed on Ascentis C18, 150 × 0.46 mm column with isocratic elution of 50:50% (v/v) 0.02 M KH_2PO_4-acetonitrile at a flow rate of 0.8 ml min^{-1}. The DAD was operated at a wavelength range between 190 and 400 nm. For the quantitative determination a calibration curve for each compound was done between concentration and absorbance at maximum wavelengths (ibuprofen 196 nm and carbamazepine 214 nm). The compounds were identified based on comparison of retention time and spectral characteristics with those of standard solution.

At the end of the biodegradation experiment the amount of pharmaceuticals that adsorbed to the biomass was determined. For this purpose, the bacterial suspensions were centrifuged (2360 *g* 10 min) and the biomass (pellet) was resuspended in 5 ml HPLC grade acetonitrile. Subsequently, the resuspended biomass was sonicated three times for 30 s at 20 kHz and at an amplitude of 20% using a Branson Digital Sonifier (Emerson Industrial Automation); between two sonication steps the suspensions were vortexed for 30 s. After the ultrasound treatment (sonication), the samples were centrifuged and the supernatants were filtered (0.45 μm cellulose acetate syringe filters) to round bottom evaporating flasks (250 ml). The acetonitrile was evaporated under vacuum using an IKA® RV10 rotary evaporator (Sigma-Aldrich, Hungary) at maximum 40 °C and the residues were re-dissolved in 2.5 ml of mineral salts solution. The concentration of pharmaceuticals in the solution was determined using HPLC as described above.

Results and discussion

Hereinafter, from the whole research regarding the microbiology of DIC, IBU and CBZ biodegradation we focus particularly only on those data that are relevant to the genus *Nocardioides* and *Nocardioides* sp. nov. strain CBZ_1^T. Comprehensive evaluation and presentation of enrichment cultures' metagenome data, complete results of bacterial isolation, identification and pharmaceutical biodegradation testing of other bacterial isolates are beyond the scope of the present paper.

The relative abundance of the genus Nocardioides in the enrichment cultures as assessed by metagenome analysis

Read-based metagenomic data revealed that the genus *Nocardioides* could have a substantial role in the IBU and CBZ amended enrichment cultures. Whereas in the initial biofilm community the relative abundance of *Nocardioides* spp. was only 0.14%, in the IBU and CBZ enrichments it increased to a maximum of 2.7% (Fig. 1). Apart from *Nocardioides* spp., the IBU amended enrichments contained other genera such as *Methyloversatilis* (max. 11.5%, 2nd month), *Pseudomonas* (12.1%, 3rd month), *Methylibium* (2%, 1st month) and *Rhodococcus* (2%, 1st month); the CBZ amended enrichments contained *Pseudomonas* (max. 4.1%, 3rd month), *Methyloversatilis* (11.9%, 1st month) *Azospirillum* (4%, 2nd month), *Rhodococcus* (2.8%, 1st month), *Variovorax* (2%, 2nd month) and *Pseudonocardia* (1.5%, 3rd month).

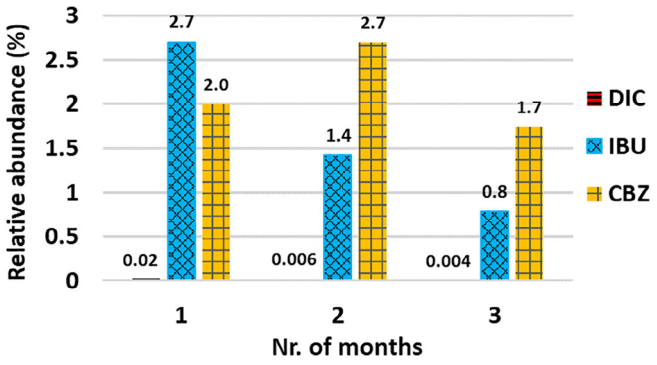

Fig. 1. The relative abundance of *Nocardioides* spp. in the enrichment cultures as assessed by shot-gun metagenome sequencing and analysis. DIC – diclofenac enrichment; IBU – ibuprofen amended enrichment; CBZ – carbamazepine amended enrichment.

On the other hand, in the DIC amended enrichments the relative abundance of the genus *Nocardiodies* remarkably decreased and varied between only 0.02 and 0.004%. (Fig. 1).

At the genus level, DIC enrichments were dominated by *Pseudomonas*, *Methyloversatilis* and *Azospirillum*, reaching a maximum of 34.5% (1st month), 8.3% (3rd month) and 3.1% (3rd month) relative abundance in at least one of the enrichment cultures, respectively.

These data suggest that most probably diclofenac sodium inhibited the growth of strain CBZ_1^T which is not surprising because the antimicrobial activity of diclofenac sodium has already been reported earlier [66–68].

Reconstruction of a Nocardioides affiliated bin genome from metagenomes, results of RPKM calculations

Genome-resolved metagenome analysis has been utilized for the recovery of bacterial genomes from the most diverse environments and provided first genome representatives of uncultivable microbes and insights into previously unexplored metabolic traits of the microbes [69]. Also, in our study, a high-quality metagenome assembled genome, representative of a yet uncultivated bacterium was obtained and affiliated to the genus *Nocardioides*; completeness 100%, genome size 6.18 Mb, GC content 71.43%).

According to the calculated RPKM values, the *Nocardioides* species corresponding to the obtained bin genome was active in the carbamazepine and ibuprofen enrichments. Compared to the initial state (RPKM values obtained for the starting biofilm sample), in the carbamazepine and ibuprofen enrichments the obtained RPKM values were up to 76 and 43 times higher, respectively. In contrast, in the diclofenac sodium amended enrichment the activity of the bin genome related *Nocardioides* sp. decreased reflected by the remarkable decrease of the RPKM value. In the case of the two- and three-months old diclofenac sodium enrichments less than one tenth of the initial RPKM value was obtained (Fig. 2).

The aforementioned findings were corroborated by the RPKM values obtained on the basis of the metatranscriptomic data of the three-months old enrichments. Also, on the basis of metatranscriptomic data the *Nocardioides* species corresponding to the reconstructed bin genome was active in the ibuprofen and carbamazepine enrichments but not in diclofenac sodium. RPKM values in the ibuprofen (2.7) and carbamazepine (2.5) enrichments were ~200 times higher than that of the diclofenac sodium enrichment (0.01) (Fig. 2C).

The results of RPKM value calculations also supported the inhibitory effect of diclofenac sodium on the growth of *Nocardioides* spp.

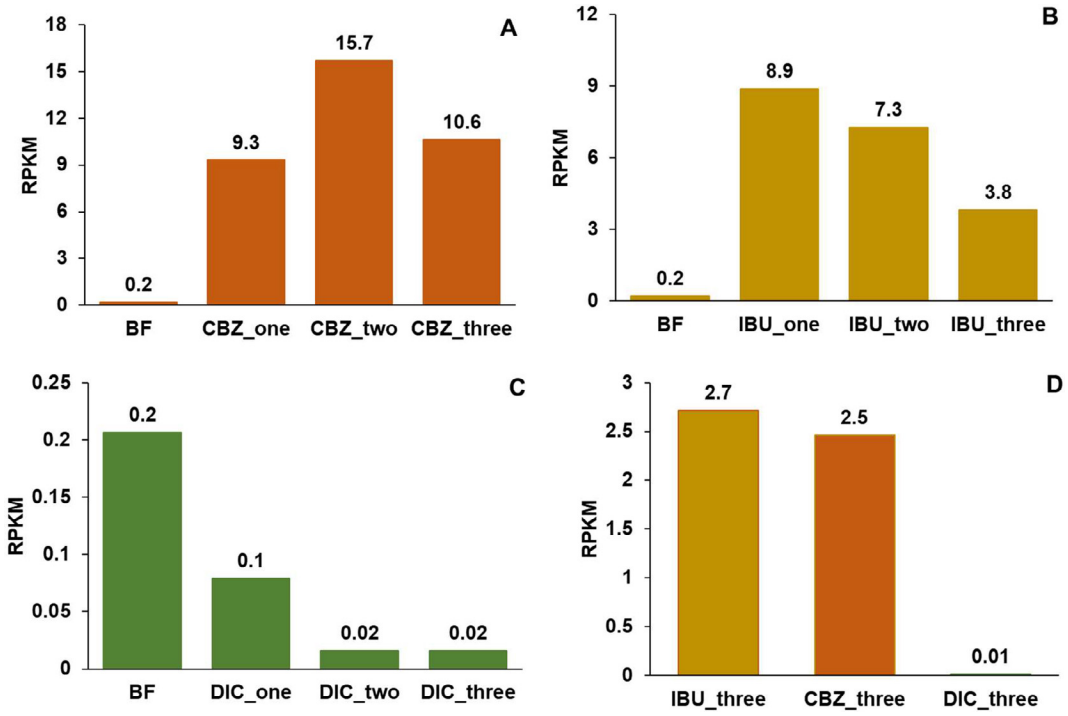

Fig. 2. RPKM values obtained in each enrichment based on metagenome (A-C) and metatranscriptome data (D). BF – initial biofilm; CBZ – carbamazepine enrichment; IBU – ibuprofen enrichment; DIC – diclofenac sodium enrichment; one, two and three – number of months.

Using sequence read recruitment the reconstructed novel *Nocardioides* sp. genome could be identified also in the metagenome of pharmaceutical amended liquid enrichment cultures where pharmaceuticals were used as the sole source of carbon and energy. The relative abundance of the novel *Nocardioides* sp. in the trimethoprim amended enrichment was nearly 9% and in one of the carbamazepine enrichments it was 3% ([51]; Table S1).

Isolation and phylogenetic characterization of Nocardioides sp. strain CBZ_1^T

The main subject of the study, the isolate *Nocardioides* sp. strain CBZ_1^T was obtained from the one-month-old carbamazepine enrichment. A continuous stretch of 1403 bp of 16S rRNA gene of strain CBZ_1^T was sequenced and taxonomic affiliation of the isolate was determined. The closest 16S rRNA gene sequences belonged to *N. kongjuensis* DSM19082^T (98.4%), *N. daeguensis* JCM17460^T (98.4%) and *N. nitrophenolicus* DSM15529^T (98.2%) (Fig. 3A, Table 2).

General features of the sequenced genome of strain CBZ_1^T are summarized in Table 1. The genome was assembled into 25 contigs. The size of the recovered genome was 6,303,422 bp with a G + C content of 71.43% that fell into the range of G + C contents of the genus *Nocardioides* [70]. Based on available genomes, the ANI and *in silico* dDDH hybridization values between the type strain CBZ_1^T and the type strains of the closest relatives ranged from 83.3 to 85.8% and 29.3 to 33.6%, respectively (Table 2). The obtained ANI and dDDH values were lower than the 95–96% ANI and 70% dDDH thresholds for species delineation [71,72].These results indicated that strain CBZ_1^T represents a distinct species of the genus *Nocardioides*.

Moreover, the generated phylogenomic trees based on 92 or 120 bacterial marker genes also supported the fact that *Nocardioides* sp. CBZ_1^T is a new phyletic lineage within the genus *Nocardioides* (Fig. 3B, Fig. S1). It has to be added that based on phylogenomic studies the closest type strain to the novel species was *N. humi* DCY24^T (Fig. 3B, Fig. S1). In addition, genome-based phylogenetic studies have shown that the reconstructed *Nocardioides* sp. bin2 genome belongs to the proposed novel *Nocardioides* species represented by isolate CBZ_1^T; both, *in silico* dDDH and ANI values were well above the cut-off recommended for the demarcation of new species (Table 2).

Compared to the closest relatives (23 *Nocardioides* spp.), on the basis of the pan-genomic study the novel *Nocardioides* species harbors 1219 unique genomic islands (UGIs; Fig. S2, Supplementary file).

It must be highlighted that it was possible to bring into culture a metabolically relevant, new to science bacterial species most probably involved in carbamazepine and ibuprofen degradation, whose genome was first reconstructed by using genome-resolved metagenomics. To the best of our knowledge, in the literature there are no other studies reporting the simultaneous isolation and genome reconstruction of a novel bacterial species. The present study confirms the applicability of metagenome binning for the exploration of the unknown bacterial diversity. Further evidence was obtained regarding the biological reality of reconstructed bacterial genomes a topic thoroughly discussed also in the review of Sebutal [74].

Morphological characteristics of Nocardioides sp. CBZ_1^T

Isolate CBZ_1^T had Gram-positive, elongated coccoid cells measuring 0.8–1.2 μm in length and 0.4–0.6 μm in width. No motility was observed. Transmission electron microscopy of negatively stained cells showed no presence of flagella (Fig. 4). When grown on R2A agar, the isolate forms whitish, translucent, flat colonies with irregular margins.

Chemotaxonomic characteristics

The major respiratory quinone of strain CBZ_1^T was identified as tetrahydrogenated menaquinone with eight isoprene units,

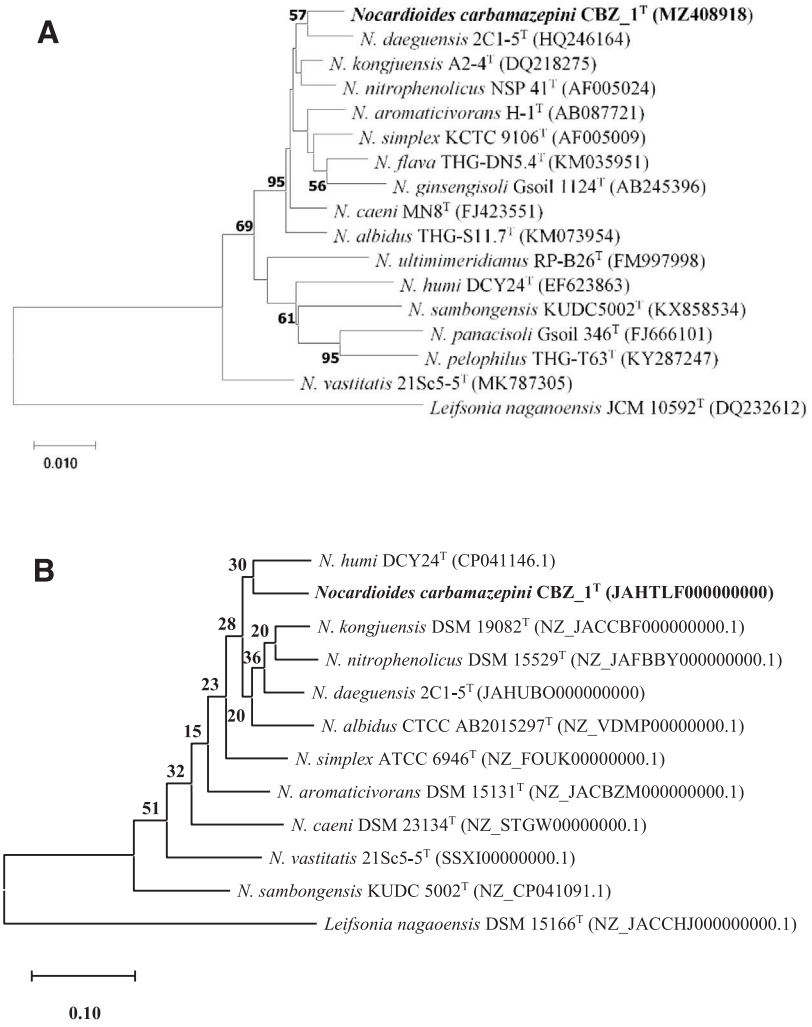

Fig. 3. (A) Phylogenetic tree based on *16S rRNA* gene sequences showing relationship between strain CBZ_1^T and the closest relatives within the genus *Nocardioides*. The scale of 1% nucleotide substitution rate and the bootstrap values with 10,000 re-samplings >50 are shown at branching points. (B) Phylogenetic tree inferred using UBCGs (concatenated alignment of 92 core genes). Gene support indices (GSIs) are given at branching points. Bar, 0.1 substitution per position. For the construction of phylogenetic trees, the *16S rRNA* gene and whole genome sequence of *Leifsonia naganoensis* was used as an outgroup to root the trees. The accession numbers of the used sequences are given in parentheses.

Table 1
General features of the genome of *Nocardioides sp.* strain CBZ_1^T.

Characteristic	Value
checkM* Completeness (%)	98.5 (4 marker genes are missing)
checkM contamination (%)	0.8 (2 marker genes are duplicated)
Size (bp)	6,303,422
G + C content (mol%)	71.4
Total number of genes	6,407
Number of protein coding sequences	6,328
Number of pseudogenes	2
Average gene length (bp)	956
rRNA (16S, 23S, 5S)	5
tRNA genes	45
GenBank accession number (NCBI)	JAHTLF000000000

*CheckM embedded in the MicroScope platform is an automated method for assessing the quality of a microbial genome regarding completion and contamination [73].

MK8 H4 (98.8%). The major polar lipids were phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) (Fig. S3). Both the major respiratory quinones and polar lipids are typical of *Nocardioides* spp. [75].

The total hydrolysate (100 °C, 4 N HCl, 16 h) of the peptidoglycan contained muramic acid (Mur) and the amino acids diaminopimelic acid (Dpm), alanine (Ala), glycine (Gly) and glutamic acid. Quantification of amino acids by GC/MS or N-heptafluorobutyric amino acid isobutylesters resulted in the following molar ratio: 2.6 Ala, 1.4 Gly, 1.0 Glue, 1–0 Glu: 0.8 Dpm. The analysis of enantiomers of the total hydrolysate showed the presence of LL-Dpm. The peptides Mur-Ala, Mur-Ala-Glu, Glu-Dpm, Gly-Ala and a minor amount of DPm-Gly-Ala-Dpm could be detected after hydrolysis under milder conditions (4 N HCl, 45 min., 100 ℃). Based on the before mentioned the occurrence of the following peptidoglycan type was concluded: A3y LL-DpM-Gly.

The cellular fatty acid profiles of the investigated strains are summarized in Table 3. The major components for strain CBZ_1^T were 10Me $C_{18:0}$ (20.7%), $C_{18:1}$ ω9c (15.9%), $C_{16:0}$ ISO (10.4%) $C_{17:0}$ ISO (9.7%) and $C_{17:1}$ ω6c (8.0%).

Physiological and biochemical characteristics

Growth of strain CBZ_1^T occurred in a pH range of 6–9 with an optimum at pH 8. The temperature range for growth was 15–37 °C

Table 2
Phylogenetic relatedness between strain CBZ_1^T and the type strains of previously established *Nocardioides* species based on whole genome sequences. Whole genome sequence accession numbers are given in parentheses.

	Nocardioides sp. strain CBZ_1^T			
	16S rRNA gene sequence similarity (%)	ANI (%)	dDDH (%)	Mol% G + C
N. kongjuensis DSM 19082^T (NZ_JACCBF000000000.1)	98.4	83.3	29.7	72.1
N. daeguensis JCM 17460^T (JAHUBO000000000)	98.4	85.5	30.1	74.9
N. nitrophenolicus DSM 15529^T (NZ_JAFBBY000000000.1)	98.2	85.1	29.3	71.4
N. humi DCY24^T (CP041146.1)	96.5	85.8	33.6	71.4
Nocardioides sp. bin2 (JAJTIU000000000)	-*	99.9	99.4	71.4

*no *16S rRNA* gene was detected in the reconstructed *Nocardiodes* sp. bin2

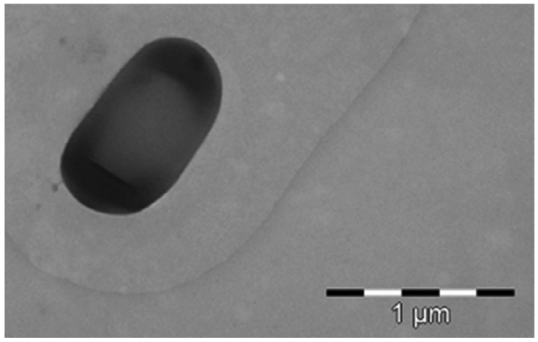

Fig. 4. Cell morphology of strain CBZ_1^T observed by transmission electron microscopy with negative-staining.

with the optimum at 28 °C. NaCl tolerance ranged between 0 and 3%, with the optimum of 1%.

Physiological and biochemical characteristics, and enzymatic activity of strain CBZ_1^T compared to those of the related type strains are shown in Tables 4 and 5. Strain CBZ_1^T was oxidase negative and catalase positive, reduced nitrate to nitrite, produced H_2S from cysteine, did not produce indole and did not ferment glucose. The isolate did not hydrolyze arginine, urea and starch, but did hydrolyze aesculin, gelatin, casein and Tween 80. It was able to assimilate only malic acid and D-glucose out of the 49 tested compounds found in API® 50CH test strips. According to the results of the API® ZYM, the strain showed alkaline phosphatase, esterase, esterase lipase, lipase, leucine-, valine-, and cysteine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and β-glucosidase, but not α-chymotrypsin, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase and α-mannosidase activity (Table 5).

Table 3
Cellular fatty acid profiles of strain CBZ_1^T and related species. Taxa: **1.** strain CBZ_1^T; **2**. *N. kongjuensis* DSM 19082^T; **3**. *N. nitrophenolicus* DSM 15529^T; **4**. *N. daeguensis* JCM 17460^T. Data are expressed as percentages of total fatty acids. -, Not detected. Fatty acids which were lower than 1.0% in all strains are not shown. All data are from the present study.

Fatty acid	**1**	**2**	**3**	**4**
Saturated				
$C_{16:0}$	4.44	1.81	2.10	1.99
$C_{17:0}$	1.03	0.59	1.09	0.56
$C_{18:0}$	3.26	3.58	-	3.50
Unsaturated				
$C_{17:1}$ ω6c	**8.00**	**9.18**	**7.12**	**11.02**
$C_{17:1}$ ω8c	2.17	2.04	2.24	2.11
$C_{18:1}$ ω7c	1.87	1.69	1.98	1.88
$C_{18:1}$ ω9c	**15.90**	**16.52**	**19.63**	**19.59**
Branched-chain fatty acids				
iso-$C_{15:0}$	3.38	1.67	2.65	1.56
iso-$C_{16:0}$	**10.42**	**22.27**	**11.98**	**23.72**
iso-$C_{16:1}$H	1.89	1.77	0.83	1.28
iso-$C_{17:0}$	**9.73**	**8.02**	**12.10**	**7.34**
anteiso-$C_{17:0}$	2.04	4.22	6.49	2.87
iso-$C_{18:0}$	0.59	2.63	1.28	3.12
10-Methyl fatty acids				
$C_{16:0}$	2.18	1.60	1.63	1.30
$C_{17:0}$	3.08	3.80	3.24	3.61
Hydroxy fatty acids				
$C_{16:0}$ 2-OH	2.45	0.57	1.26	0.69
Summed feature[a]	4.48	1.89	2.53	1.94
TBSA[b] 10Me $C_{18:0}$	**20.72**	**13.45**	**18.96**	**9.09**

[a] Summed features represent groups of two or three fatty acids that could not be separated by gas–liquid chromatography with the MIDI system. Summed features: 3, $C_{16:1}$ ω7c/$C_{15:0}$ ISO 2-OH.
[b] Tuberculostearic acid.

Table 4
Physiological and phenotypic characteristics of strain CBZ_1^T compared to the closely related species within the genus *Nocardioides*. Strains: **1**. strain CBZ_1^T; **2.** *N. kongjuensis* DSM 19082^T; **3**. *N. nitrophenolicus* DSM 15529^T; **4.** *N. daeguensis* JCM 17460^T.

Characteristics	1	2	3	4
Reduction of nitrate to nitrite	+	-	–	+
H_2S production	+	+	+	+
Oxidase	–	–	w	+
Catalase	+	+	+	+
Indole production	–	–	–	–
Fermentation of glucose	–	–	–	–
Hydrolysis of				
Arginine	–	–	–	–
DNA	+	+	+	+
Urea	–	–	+	–
Aesculin	+	+	+	–
Gelatin	+	+	+	+
Casein	+	+	–	+
Tween 80	+	+	+	+
Starch	–	+	+	+
Assimilation of				
D-glucose	+	+	+	–
L-arginine	–	–	–	–
L-arabinose	–	–	w	–
D-mannose	–	–	w	–
D-mannitol	–	w	w	–
N-acetyl-glucosamine	–	w	w	–
D-maltose	–	w	w	–
Potassium gluconate	–	w	w	–
Capric acid	–	–	–	–
Adipic acid	–	w	w	w
Malic acid	+	w	w	w
Trisodium citrate	–	w	w	–
Phenylacetic acid	–	–	–	w
Acid production				
D-maltose	–	–	–	–
Mannose	–	w	w	–
Ribose	–	–	+	+
D-fructose	–	–	+	–
D-glucose	–	–	–	w
Lactose	–	–	–	–
L-arabinose	–	–	–	–
Cellobiose	–	–	–	–
Xylose	–	–	+	+

Symbols: +, positive; –, negative; w, weak positive.

Table 5
Enzymatic activity of strain CBZ_1^T and the closest relatives as revealed by API® ZYM test (bioMérieux SA, France). Strains: 1. strain CBZ_1^T; 2. *N. kongjuensis* DSM 19082^T; 3. *N. nitrophenolicus* DSM 15529^T; 4. *N. daeguensis* JCM 17460^T.

Enzyme assay	**1**	**2**	**3**	**4**
Alkaline phosphatase	+	+	+	+
Esterase (C 4)	+	+ (–[a])	+ (–[b])	+
Esterase lipase (C 8)	+	+	+	+
Lipase (C 14)	+	–	–	–
Leucine arylamidase	+	+	+	+
Valine arylamidase	+	+ (–[a])	+	+
Cystine arylamidase	+	+ (–[a])	+	+
Trypsin	+	+ (–[a])	+	+
α-chymotrypsin	–	–	–	–
Acid phosphatase	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+
α-galactosidase	–	–	–	–
β-galactosidase	+	w (–[a])	w (+[b])	w (+[c])
β-glucunoridase	–	–	–	–
α-glucosidase	+	+	+	+
β-glucosidase	+	+ (–[a])	w	w (+[c])
N-acetyl-β-glucosaminidase	–	–	–	–
α-mannosidase	–	–	–	–
α-fucosidase-fucosidase	–	–	–	–

Symbols: +, positive; –, negative; w, weak positive.
[a] Data for the type strain of *N. kongjuensis* DSM 19082^T from[76].
[b] Data for the type strain of *N. nitrophenolicus* DSM 15529^T from[21].
[c] Data for the type strain of *N. daeguensis* JCM 17460^T from[25].

Pharmaceutical biodegradation capacity of strain CBZ_1^T

Contrary to our expectations, under the tested conditions strain CBZ_1^T showed no remarkable carbamazepine biodegradation. Only a slight carbamazepine concentration reduction of 4%, was detected after 7 weeks of incubation, in the presence of yeast extract (0.3 g l^{-1}) as co-substrate (data are not shown). These findings contradict the results of metagenomic studies which showed that strain CBZ_1^T was active also in the carbamazepine enrichment. It can be speculated that most probably the new species was not directly involved in the initial attack of carbamazepine but in the biotransformation of carbamazepine metabolites originating from the catabolic activity of other bacteria. It has to be noted that carbamazepine is an emerging contaminant that is resistant to biodegradation. Only a few pure bacterial isolates, such as *Labrys portucalensis* F11 and *Streptomyces* MIUG 4.89, have been obtained so far capable of carbamazepine biodegradation [77,78].

A significant biodegradation was detected in the presence of ibuprofen. After 7 weeks of incubation, in the presence of glucose (3 g l^{-1}) as co-substrate, 70% of ibuprofen was eliminated from the test solutions by strain CBZ_1^T (Fig. 5). Analyses showed no ibuprofen adsorption to the biomass. Although *Nocardioides* spp. are well known due to their xenobiotic biodegradation capabilities (dibenzofuran, nitro- and chlorophenols, carbendazim, pesticides, PAHs etc.) no *Nocardioides* strain has been reported so far capable of ibuprofen biodegradation [19–21,25–31]. It has to be added that ibuprofen degrading bacterial strains affiliating to other genera, e.g., *Sphingobium*, *Sphingomonas*, *Patulibacter* and *Bacillus*, do exist in general [79–82]. No better or higher degradation rates could be reached in the case of other experimental settings such as in the presence of other co-substrate or under lower co-substrate concentrations.

Based on whole-genome sequencing, gene annotations and metabolic analyses it was found that isolate CBZ_1^T encodes functional genes involved in xenobiotics biodegradation including pharmaceutical compounds (Table S2). Genes similar to biodegradative genes involved in ibuprofen biodegradation (*ipf*-DEFG), first identified in the case of *Sphingomonas* sp. Ibu-2 [80], could also be identified, however gene sequence similarities ranged only between 33 and 50% (Table S2.) Among the identified UGIs, genes involved in xenobiotics biodegradation such as 1,2-phenylacetyl-CoA epoxidase involved in phenylacetate degradation; acetyl-CoA acetyltransferase involved in benzoate and butanoate degradation; 2-keto-4-pentenoate hydratase involved in benzoate, dioxin and xylenes degradation; aldehyde dehydrogenase involved in chloroalkane and chloroalkene degradation; alcohol dehydrogenase involved in chloroalkane and chloroalkene degradation; naphthalene degradation, metabolism of xenobiotics by cytochrome P450 pathway (trichloroethylene, felbamate, cyclophosphamide); phenylpropionate dioxygenase involved in aromatic compound metabolism, catechol 2,3-dioxygenase system involved in the ring fission of aromatic compounds could be identified (Supplementary file). A cytochrome P450 enzyme (CYP45) encoding gene – *Cyp*X (PDB:3A4Z) – was also identified as unique for the novel *Nocardioides* species. This finding is interesting since CYP45 enzymes are thought to be responsible for carbamazepine biodegradation at least in the case of white-root fungi such as *Trametes versicolor* and *Pleurotus ostreatus* [83,84]. It may be speculated that this type of CYP45 encoding gene, unique amongst the analyzed 23 *Nocardioides* spp., might be responsible for the activity and proliferation of the novel species in the carbamazepine amended enrichments.

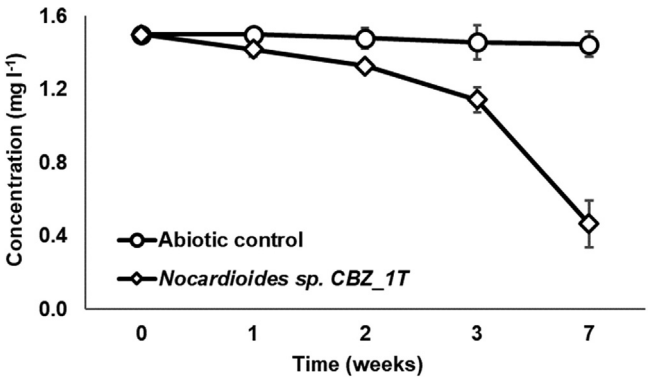

Fig. 5. The biodegradation rate of ibuprofen by *Nocardioides* sp. strain CBZ_1^T.

Table 6
Description of *Nocardioides carbamazepini* sp. nov.

Genus name	***Nocardiodies***
Species name	*Nocardioides carbamazepini*
Specific epithet	*carbamazepini*
Species status	sp. nov.
Species etymology	car.ba.ma.ze.pi'ni. N.L. neut. n. *carbamazepinum*, carbamazepine; N.L. gen. n. *carbamazepini*, of carbamazepine
Description of the new taxon and diagnostic traits	Cells are Gram-positive, strictly aerobic, non-motile, the elongated coccoid cells are 0.8–1.2-μm in length and 0.4–0.6 μm in width. The colonies are flat and whitish, translucent with irregular margins on R2A agar. Grows well at 15–37 °C with an optimum at 28 °C. Grows at pH 6–9, with the optimum growth at pH 7–8. Growth occurs in the absence of NaCl and in the presence of up to 3% (w/v) NaCl. Grows well in R2A, nutrient, LB and TSA broth, showing the best growth in R2A. Catalase positive and oxidase negative. Reduces nitrate to nitrite, but does not reduce nitrate to dinitrogen gas. Tests for alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase and protease are positive; tests for α-chymotripsin, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α- mannosidase, α-fucosidase, arginin dihydrolase, urease, indole production, acidification of glucose are negative. Except assimilation of malic acid and glucose, it is negative for assimilation of other carbon sources found in API® 20NE and API® 50CH test strips. MK8H4 is the predominant respiratory quinone, the type of peptidoglycan is A3y LL-Dpm-Gly and the major cellular fatty acid are TBSA 10Me $C_{18:0}$ (20.7%), 18:1 w9c (15.9%), 16:0 ISO (10.4%) 17:0 ISO (9.7%) and 17:1 w6c (8.0%).
Country of origin	Hungary
Region of origin	Central Region of Hungary
Date of isolation	19/01/2020
Source of isolation	Biofilm bacterial community selectively enriched on carbamazepine for 1 month in mineral salts solution
Sampling date	18/12/2019
16S rRNA gene accession nr.	MZ408918
Genome accession number	JAHTLF000000000
Genome status	Incomplete (draft)
Genome size	6,303 kbp
GC mol%	71.43
Number of strains in study	1
Information related to the Nagoya Protocol	Not applicable
Designation of the Type Strain	CBZ_1^T
Strain Collection Numbers	NCAIM B.0.2663 = LMG 32395

Conclusions

One of the most interesting findings and also the uniqueness of this study is that using metagenome binning the genome of a biotechnologically important, novel bacterial species could be reconstructed and at the same time the same novel bacterial species could also be brought into cultivation. To the best of our knowledge, such report has not been published before. Nowadays using metagenome binning hundreds of thousands of new bacterial genomes are described on a "daily basis" from metagenomes (MAGs), e.g. from gut microbiomes [85–88] etc., however without being able to cultivate those bacteria. Without conventional cultivations the reliability of the method in the reconstruction of novel bacterial genomes is questionable, and the question arises whether the obtained MAGs are real or are simply "chimeras" of different bacterial genomes? [74]. This study provides further evidence that metagenome binning is a working approach to have access *in silico* to genomes of real, new to science bacterial species and is a suitable way to explore the microbial dark matter.

The polyphasic analyses based on phenotypic and chemotaxonomic data, supplemented with phylogenetic and genomic analyses, indicated that the obtained bacterial isolate capable of ibuprofen biodegradation, obtained from a carbamazepine amended selective enrichment culture, represents a novel species within the genus *Nocardioides*, and the name *N. carbamazepini* sp. nov. is proposed, with strain CBZ_1^T as the type strain (=NCAIM B.0.2663 = LMG 32395). The novel species description is given in the protologue Table 6.

Nucleotide accession numbers

The GenBank/EMBL/DDB accession number for the 16S rRNA gene nucleotide sequence is MZ408918. The accession number for the whole genome sequence of strain CBZ_1^T is JAHTLF000000000.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2022.126339.

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