



# Biodegradation of Paracetamol by Some Gram-Positive Bacterial Isolates

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Received: 23 August 2020 / Accepted: 17 May 2021 / Published online: 4 June 2021  
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## Abstract

Bacterial isolates with the capacity to remove paracetamol were selected from an activated sludge sample collected in an oxidation ditch of a wastewater treatment plant. Among these, twelve bacterial isolates were selected according to their capacity to grow in the presence of paracetamol. They were identified using the colony morphotype procedure and by 16S rRNA gene sequencing analysis, but only four of them showed the ability to utilise paracetamol as the sole carbon source in the presence of a nitrogen supply. Those four bacterial isolates were assigned to species of the genera *Bacillus*, [*Brevibacterium*], *Corynebacterium* and *Enterococcus*. Bacterial isolates were cultured in liquid mineral salt medium (MSM) spiked with 200 mg/L of paracetamol at 28 °C in the dark. In cultures inoculated with [*Brevibacterium*] *frigoritolerans*, *Corynebacterium nuruki* and *Enterococcus faecium*, removal of  $97 \pm 4\%$ ,  $97 \pm 6\%$  and  $86.9 \pm 0.8\%$  of paracetamol at 200 mg/L were obtained, respectively, while in the presence of a species belonging to *Bacillus cereus* group removal of the drug below the limits of detection was attained with evidence of mineralisation, after 144 h of incubation. During the degradation process, the metabolites 4-aminophenol, hydroquinone and 2-hexenoic acid were detected. As far as we know, these species are herein first-time described as paracetamol degraders.

## Introduction

Acetaminophen (N-acetyl-p-aminophenol), universally recognised as paracetamol, is regarded as a safe non-steroidal anti-inflammatory drug (NSAID) at therapeutic doses, which is extensively used as an analgesic and antipyretic and provides effective relief of the symptoms [1]. This drug is considered one of the most utilised worldwide, but despite its general usage, the current information about paracetamol concentrations in Wastewater Treatment Plants (WWTPs) is restricted [2].

The biodegradation behaviour and sorption of paracetamol and its metabolites can be estimated by a group of critical physicochemical properties such as the pseudo-first-order degradation constant ( $K_{\text{biol}}$ ) which ranged from 50 to

80 L/gSS.day and 106 to 204 L/gSS.day [3]. Thus, according to the literature, this drug can be considered a highly biodegradable compound, with a  $K_{\text{biol}} > 10$  L/gSS.day, an octanol–water partition coefficient ( $\log K_{\text{ow}}$ ) of 0.46 and a  $\log K_{\text{ow}} < 2.5$ , which indicates a highly hydrophilic compound [3].

Drug concentrations varied from 350 to 180,000 ng/L and 10 to 13,000 ng/L in the inflowing and outflowing wastewaters, respectively, according to the surveyed wetlands systems in Czech Republic's [2]. Kasprzyk-Hordern et al. [4] reported paracetamol concentrations ranging from 1826 to 24,525 ng/L in the effluents of WWTP's Cilfynydd (South Wales), whereas in Volos's WWTP, Greece, the concentrations of paracetamol in the effluents of aerobic tanks systems were up to 305 ng/L [5]. In Portugal, the maximal concentration of paracetamol documented in treated wastewaters was 32 µg/L [6]. Despite the high degradation rates reported in treated wastewaters compared with the raw sewage, paracetamol in the WWTP effluents at a global level recalls the necessity to implement more efficient treatment processes for its elimination [e.g. 5, 7]. Although paracetamol seems to be properly degraded during the WWTP in comparison with other drugs, it was identified in shallow waters ranging from 110 to 10,000 ng/L [8], e.g. in the river

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Taff, United Kingdom, it was found in concentrations from 305 to 1534 ng/L [4] and in USA's natural hydric resources at concentrations up to 10 µg/L [9]. Additionally, during an investigation at the national level to track hazardous organic compounds in Australian rivers, paracetamol was one of the most detected contaminants at high concentrations of 7150 ng/L [10]. Another sign that this pollutant should be of global concern is because it was detected in high-sea waters, such as in the western Mediterranean Sea, where paracetamol was found at concentrations between 0.468 and 1.70 ng/L [11, 12].

The screening of paracetamol and its metabolic products in shallow waters indicates that the extend of human usage and release of the drug surpass their actual elimination by the conventional WWTP [13]. The metabolic products can persist and be even more hazardous than the original compound. Thus, the need to investigate them [13–15] and seek novel and successful approaches to eliminate them from sewage and treated wastewaters [12].

For this purpose, biodegradation can signify an effective solution regarding environmental and economic aspects [14].

Bacteria have been demonstrated to perform a critical role in the biodegradation of organic compounds in WWTPs.

In the case of paracetamol, several bacteria with the capacity to use this drug as carbon and energy supply have been isolated; some authors suggested metabolic pathways for paracetamol biodegradation [16–18]. For example, Zhang et al. [17] isolated strains from the genera *Stenotrophomonas* and *Pseudomonas* from microbial clusters and Karaman et al. [18] found that *Pseudomonas aeruginosa* biodegraded the drug from the activated sludge in less than a month. They proposed a metabolic pathway for paracetamol biodegradation, having as first metabolites 4-aminophenol and hydroquinone [17, 18]. More recently, Abdullah et al. [19] found *Pseudomonas aeruginosa* strains signed as STB2 and STB4 with the ability to degrade 79.4% and 88.4% of 3000 mg/L of paracetamol, respectively, in 120 h.

This work focuses on the search for microbial isolates with the ability to biodegrade paracetamol. For that purpose, bacteria were isolated from sludge containing an aerobic community used in previous studies performed by our research group [12], which proved to biodegrade paracetamol.

## Material and Methods

### Source of Inoculum and Culture Preparation

For this study, a sample of aerobic activated sludge was collected from the oxidation ditch of Faro Northwest WWTP's, Portugal, to test the paracetamol biodegradability by

bacterial isolates. This sludge was previously used in studies aiming to select an aerobic community with the ability to degrade paracetamol [12]. The sludge washing procedure was described by Palma et al. [12].

### Bacterial Isolates From an Aerobic Community

The pellet resulting from the sludge previously washed was incubated in 9 mL of 0.1% peptone water at 28 °C for 2 h. A successive dilutions protocol was performed by adding 1 mL of the original culture in 9 mL of broth to each tube. Subsequently, 100 µL of each dilution was spread onto the solid plate cultures with mineral salt medium (MSM) a medium without carbon compounds which was composed by 0.5 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.5 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.01 g/L NaCl, 0.2 g/L MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.02 g/L CaCl<sub>2</sub>, 0.0004 g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.0004 g/L CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.0003 g/L MnSO<sub>4</sub>, 0.01 g/L ethylenediaminetetraacetic acid (EDTA) and 0.0003 g/L (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, with a nitrogen source of 1 g/L (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> and agar (15 g/L). The experiments were carried out adding to the solid MSM:

- i. 10 g/L glucose, as a positive control, to monitor bacterial growth, given that this compound is the preferred carbon source of most of the heterotrophic bacteria;
- ii. 200 and 500 mg/L of paracetamol as a unique carbon source.

The isolates which grew in the presence of 500 mg/L of paracetamol were cultured at a lower concentration of the drug (200 mg/L).

Two main explanations can be given on why high paracetamol concentrations above those found in the environment were used in the present work. The resistance of bacteria to such high concentrations was studied as well as its biodegradation. In this study, paracetamol was used as the sole carbon source, thus requiring such high concentrations to ensure that there was enough carbon source to allow the optimal growth of bacteria. Other authors performed their experiments using very high concentrations of paracetamol, such as, for example, Zhang et al. [17] and Abdullah et al. [19]. In addition, according to the BioWin predictive model, it can be assumed that if bacteria can resist and degrade higher concentrations of the drug, they may be able to use it as carbon or/and energy source even in a complex medium such as wastewater [20].

These cultures performed in a solid medium allowed the selection and isolation of bacteria resistant to paracetamol. The bacterial were selected, picked, and purified as described in [21–23] for further identification and to perform biodegradation experiments.

A positive reaction for catalase activity was evaluated through the monitorisation of bubble generation due to the release of oxygen after applying a 3% (v/v) hydrogen peroxide solution. Oxidase activity was determined with Microbact oxidase strips (OXOID).

The gram staining protocol was accomplished and observed on a light microscope (Leica Microsystems).

## Identification of Isolates That Degrade Paracetamol

### DNA Extraction and Amplification of 16S rRNA Gene

The biomass production for DNA extraction was described in Palma et al. [23]. To identify bacterial isolates putatively capable of degrading paracetamol, DNA was extracted from each isolate bacterial culture with the PowerSoil® DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) following the protocol [12].

The 16S rRNA gene was amplified from the DNA samples extracted from the bacterial isolates through the polymerase chain reaction (PCR). The conditions used to perform the PCR amplification of the bacterial 16S rRNA gene conserved region are described in Palma et al. [23].

### DNA Sequencing

The obtained isolates were further identified by DNA of 16S rRNA gene amplification (originating a purified PCR product of 1484 bp). Direct sequencing of the PCR products was carried out by the Sanger method as described in Palma et al. [23]. The results were obtained by Sequencing Genetic Analysis Software, provided by CCMAR's Molecular Biology platform [23].

The sequence reads analysis were treated using the BIOedit Sequence Alignment Editor, and contig-0 sequences were generated, and taxonomic identification was established through Blast search of Gene Bank database of NCBI and RDP Classifier and the obtained isolates were identified. The multiple-sequence alignment was performed in MEGA-X version 10.2.4 [24] using the ClustalW package. Tamura-Nei model was used as nucleotide substitution method with discrete Gamma distribution [25], while the phylogenetic tree analysis was performed using the maximum likelihood with 500 bootstrap replications. The phylogenetic analysis of the obtained bacterial isolates was carried out by comparing their 16S rRNA gene sequences with the type strain. The information about type strains was listed at BacDive—the Bacterial Diversity Metadatabase as stated in the DSMZ (<https://www.dsmz.de/>): species: *Bacillus pacificus*<sup>T</sup> with BacDive ID: 140961; *Bacillus tropicus*<sup>T</sup> with BacDive ID: 140962; *Bacillus paranthracis*<sup>T</sup> strain with BacDive ID: 140960; species: *Bacillus cereus*<sup>T</sup> with BacDive ID: 624;

species: *Brevibacterium frigiditolerans*<sup>T</sup> with BacDive ID:1846; species: *Bacillus simplex*<sup>T</sup> (*Peribacillus simplex*<sup>T</sup>) with BacDive ID: 790; species: *Corynebacterium nuruki* S6-4<sup>T</sup> with a record number: 789493 (<https://lpsn.dsmz.de/species/corynebacterium-nuruki>) and species: *Enterococcus faecium*<sup>T</sup> with BacDive ID: 5301.

Nucleotide sequence accession numbers. The 16S rDNA sequences of the four paracetamol-degrading strains have been deposited in the GenBank database under accession numbers: isolate 1 (MW817082), isolate 3 (MW817083), isolate 4 (MW817084) and isolate 9 (MW817085).

### IC<sub>50</sub> of Paracetamol for Bacterial Growth and EC<sub>50</sub> Estimation

The half-maximal inhibitory concentration (IC<sub>50</sub>) for bacterial growth is designated as the concentration of paracetamol that causes a decline of 50% in the bacteria growth (viability), and the half-maximal effective concentration (EC<sub>50</sub>) of a progressive dose–response is characterised by the concentration of paracetamol that induces 50% of its maximal effect, after a particular exposure time. These parameters were estimated by carrying out duplicate batch cultures inoculated with approximately 10<sup>7</sup> colony forming units (CFU)/mL of bacterial cells in nutrient broth spiked with paracetamol at concentrations ranging from 200 to 6000 mg/L of drug and without paracetamol (as a positive control, therefore a reference for “typical” bacterial growth) during an incubation period of 48 h at 28 °C in the dark.

The bacterial growth for IC<sub>50</sub> determination was analysed by measuring the optical density (OD) at 600 nm using a Hach-Lange spectrophotometer DR-2800 (Sköndal, Sweden). The percentage bacterial viability was calculated with the ratio of the obtained values of OD<sub>600</sub> in a culture of a specific drug concentration over OD<sub>600</sub> in the culture without, at different tested paracetamol concentrations [12]. IC<sub>50</sub> and EC<sub>50</sub> values were estimated using GraphPad Prism 9 Software using dose–response curve log (inhibitor) vs. normalised response-variable slope and log (agonist) vs. normalised response-variable slope analyses, respectively. The bacterial viability percentage (%) vs. the logarithm of paracetamol concentration (mg/L) were plotted, and the best fitting sigmoidal curves were used to calculate IC<sub>50</sub> values.

### Paracetamol Biodegradation Assays

The bacteria that grew in paracetamol were isolated in Plate Count Agar (PCA) medium for further use. After bacterial isolates identification, paracetamol biodegradation experiments were carried out in liquid medium using the MSM, supplemented with a nitrogen source of 1 g/L of (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> and a carbon source: 10 g/L glucose (positive

control) and spiked with 200 mg/L of paracetamol. Cultures with MSM and EDTA as sole carbon supply were performed for each isolate to verify if the concentration of EDTA present in the medium serves as a carbon or energy source for the isolated bacteria.

The addition of bacterial isolates to perform the cultures for biodegradation assays were carried out following the McFarland scale; thus, the initial solutions presented an absorbance at 600 nm of approximately 0.128 after 24 h, which correspond to about  $3 \times 10^8$  CFUs. Then, to obtain a  $10^6$  CFU/ml suspension, a dilution of 1:300 (v/v) was made for each bacterial isolate that displayed the ability to grow in the presence of paracetamol.

Negative controls only with the drug and in the absence of the bacterial isolates were performed. The bacterial isolate cultures were incubated at 28 °C and 150 rpm in the dark to avoid paracetamol photodegradation. The sampling was carried out after 48 h and 144 h, time intervals chosen due to the slow growth presented by these bacteria in the studied conditions. The assays were performed in triplicate. OD measurements of the bacterial growth at 600 nm were performed in a Hach-Lange™ DR 2800 spectrophotometer (Sköndal, Sweden).

#### Identification of Paracetamol Metabolic Products

Paracetamol (99% purity), 4-aminophenol (97% purity), hydroquinone (99% purity), benzoquinone ( $\geq 98\%$ ), 2-hexenoic acid (99%) and 4-nitrophenol (99% purity) used for standard solutions were purchased from Sigma-Aldrich. The concentration ranges of paracetamol standards were from 1 to 600 mg/L of the drug.

The collection, treatment and storage of samples and further analysis of paracetamol was set as described in [12].

HPLC analysis was carried out with an isocratic method using a mobile phase consisted of ACN:water (25:75, v/v) adjusted to pH 3.74 with orthophosphoric acid (85%) using a flow rate of 1.0 mL/min and a run time of 5 min with the column maintained at room temperature to analyse the biodegradation of paracetamol. The injection volume was 20  $\mu$ L, and a wavelength of 244 nm was used for detection [12].

Preliminary detection and identification of the putative paracetamol's metabolites were performed. The metabolic products known to be generated from the degradation of this drug were used as standards to verify their existence in the samples from the experiments in which the highest degradation of paracetamol was observed. According to a method already used by the research group [12], this analysis was performed with the mobile phase potassium-phosphate-buffer (pH 4.88): methanol and the following gradient program with respective mobile phase ratios (v/v): first ramp from 0 to 8 min with 80:20 to 50:50 (v/v); first stationary

step from 8 to 11 min with 50:50 (v/v); second ramp from 11 to 12 min with 50:50 to 80:20 (v/v); second stationary step from 12 to 15 min with 80:20 (v/v). The injection volume was 20  $\mu$ L, the flow rate was set at 0.8 mL/min, the column was maintained at room temperature, and the detection was performed at 244 nm [12].

## Results and Discussion

### Identification of Isolates That Degrade Paracetamol

In order to study which bacteria within the aerobic community previously studied for drug's biodegradation [12] displayed the ability for paracetamol degradation separately from that mixed culture, bacteria were isolated, and assays were performed. The optimal growth conditions were previously checked; thus, the isolates were incubated at 28 °C, which correspond to the optimal environmental conditions for biological treatments.

Colony morphotypes selected the bacterial isolates with the capacity to grow at 500 mg/L of paracetamol, a standard method in microbial ecology to isolate different species for physiological and genetic goals [21, 26].





The growth of bacteria at a  $10^{-2}$  dilution in plates with 500 mg/L paracetamol was high, with a colony count from  $6.52 \times 10^5$  to  $1.22 \times 10^6$  CFU/mL, after 48 h incubation at 28 °C in the dark. The positive control at this dilution demonstrated a higher bacterial growth in plaque ( $> 1.06 \times 10^6$  CFU/mL) after 48 h incubation at 28 °C in the dark.

From this experiment, it was possible to successfully select twelve bacteria to grow in paracetamol as a unique carbon supply. The obtained isolates were labelled as 1, 2, 3, 4 until 12. However, only four of the selected isolates maintained the capacity to grow at 500 mg/L of the drug for more than three generations; these were isolates 1, 3, 4 and 9. The study was then focused on these bacteria, whose growth is represented by the appearance of colonies in solid MSM cultures spiked with 500 mg/L of paracetamol.

The loss of resistance and ability to grow using paracetamol as a carbon source by some of the obtained isolates may be due to the consumption of carbon source and posterior accumulation of metabolic residues produced by bacteria becoming hazardous compounds in the media. Also, over this process, bacteria lose entirely their capacity to replicate. However, although some bacteria die due to unfavourable conditions, some organisms can resist and survive in the environment by generating endospores [27, 28].

Table 1 describes the morphological and biochemical features of these four bacterial isolates.

**Table 1** Morphological and biochemical features of the bacterial isolates which have maintained the capacity to use paracetamol as a unique carbon supply

Parameter	Characteristics			
	Isolate 1	Isolate 3	Isolate 4	Isolate 9
Colony morphology	Generally large colonies with a frosted-glass surface and wave margin. A central elliptical endospore is observed	Irregular/Wrinkled	Slightly irregular/Smooth	Regular/ Smooth. Pinpoint to small
Colony colour	White	Creamy	White	White
Gram reaction	Positive	Positive	Positive	Positive
Morphology	Rod/Non-Motile/Spore-forming/Single, in pairs or chains	Rod/Motile/Endospores	Rod club-shaped/Non-motile/Non-sporing/chains	Cocci/Non-motile/Non-sporing/grouped in pairs or short chains
Respiration	Facultative anaerobic	Aerobic/ Facultative anaerobic	Strictly aerobic	Facultative anaerobic
Oxidase	Positive	Negative	Positive	Negative
Catalase	Positive	Positive	Positive	Negative
Colony Aspect				

With the objective to affiliate isolates to validly named species, a maximum likelihood phylogenetic tree based on the 16S rRNA gene was constructed by comparing the almost complete 16S rRNA gene sequences of the obtained bacterial isolates to the corresponding type strain (Fig. 1).

Based on the analysis of 16S rRNA sequences, isolate 1 (MW817082) was identified as a *Bacillus cereus* group member sharing 98.68% of similarity with *Bacillus cereus* (NC\_011725.1) and *Bacillus tropicus* strain (NZ\_CP049019.1) through NCBI Blast search. As evidenced by the phylogenetic tree, isolate 1 displays very high similarity with several type strains of different species within the *Bacillus cereus* group (Fig. 1), defending the close relatedness of isolate 1 with members of this group as already described [29–31]. This group also presented high similarity with the represented species of the genus *Bacillus* (Isolate 3). Their chemotaxonomic characteristics are also related to those of the *Bacillus cereus* group, although the morphological and biochemical profiles fit more specifically with those displayed by *Bacillus Pacificus* and *Bacillus paranthracis*.

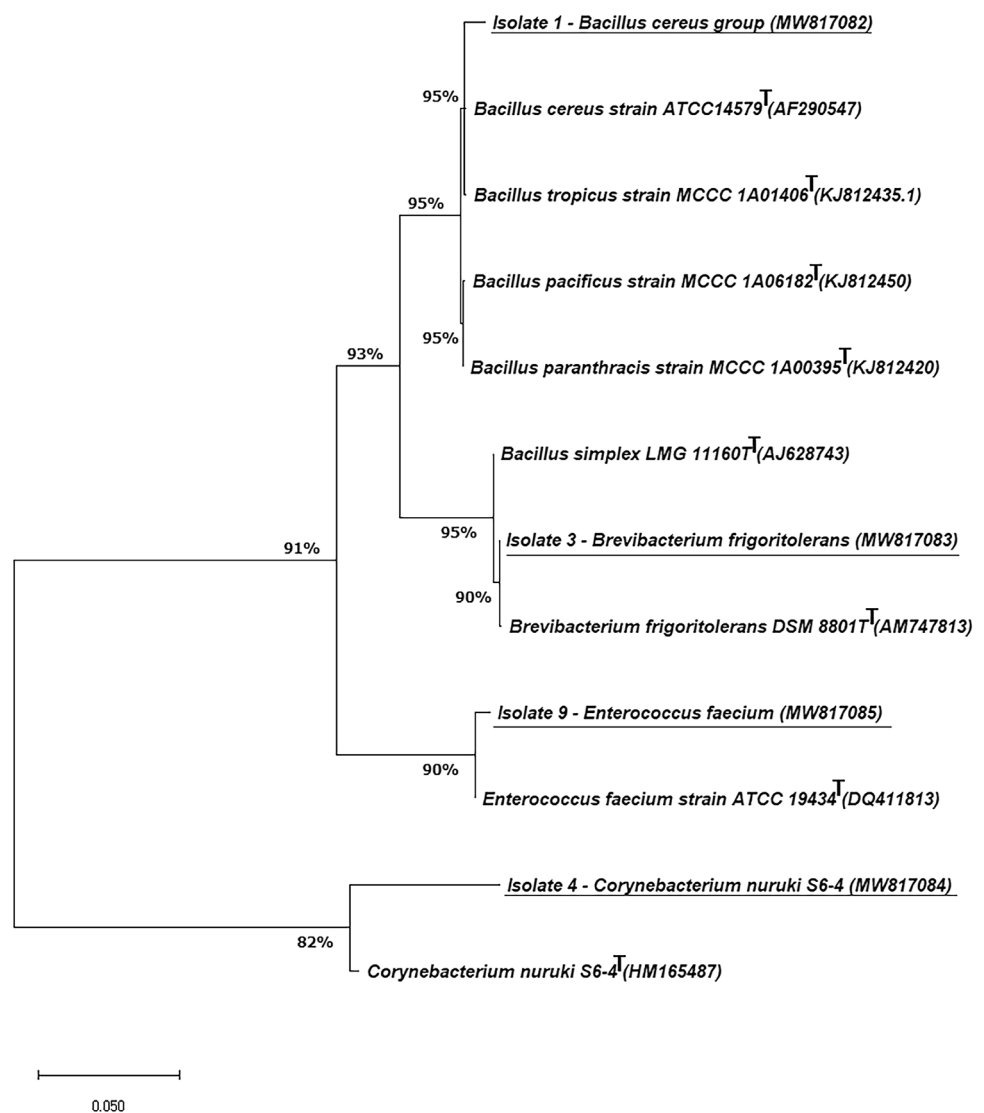
It is known that *Bacillus cereus* group species cannot be defined based only on 16S rRNA gene sequences [32], being necessary, for example, to perform a *rpoB* gene analysis to enable a more discriminatory result [31]. However, the focus of the present work is to search for bacteria that can be used in paracetamol degradation in WWTPs. Therefore, due to

the uncertainty of the exact species, isolate 1 is, herein, named as *Bacillus cereus* group.

The bacterial isolate 3 (MW817083) presented a high similarity of 16S rRNA sequences (99.86%) with a strain identified as [*Brevibacterium*] *frigoritolerans* (NR\_117474.1) through NCBI Blast search. According to the phylogenetic tree, isolate 3 assigned to [*Brevibacterium*] *frigoritolerans*, revealed to be closer to *Bacillus simplex*<sup>T</sup> than to *Brevibacterium frigoritolerans*<sup>T</sup> (Fig. 1). This result agrees with the literature that describes this specific bacterial strain as having a controversial classification. Gelsomino et al. [33] and Beesley et al. [34] referred to *B. frigoritolerans* as a misclassified *Brevibacterium* species, which do not pertain to *Brevibacterium* but which seems to belong to the genus *Bacillus*. Another aspect that supports this statement is the high similarity (>90%) shown by isolate 3 in relation to isolate 1.

Isolate 4 (MW817084) was identified with a high percentage of similarity (99.18%) with *Corynebacterium nuruki* S6-4 (NR\_117816.1) by the NCBI BLAST database, while the phylogenetic analysis also showed a good similarity with *Corynebacterium nuruki* S6-4<sup>T</sup>, showing that they are closely related. However, isolate 4 presents 91% of similarity with the other isolate's groups. Isolate 9 (MW817085) was assigned to *Enterococcus faecium* (NR\_113904.1) (99.51%) by the NCBI BLAST database and, according to

**Fig. 1** Maximum likelihood phylogenetic tree positions analysis based on the 16S rRNA gene sequences of the four bacterial isolates (*Bacillus cereus* group; [*Brevibacterium*] *frigoritolerans*; *Corynebacterium nuruki* and *Enterococcus faecium*) with the capacity to grow in the presence of paracetamol as a unique carbon source compared with each type strains. Node numbers represent bootstrap values (percentage of 500 replications)

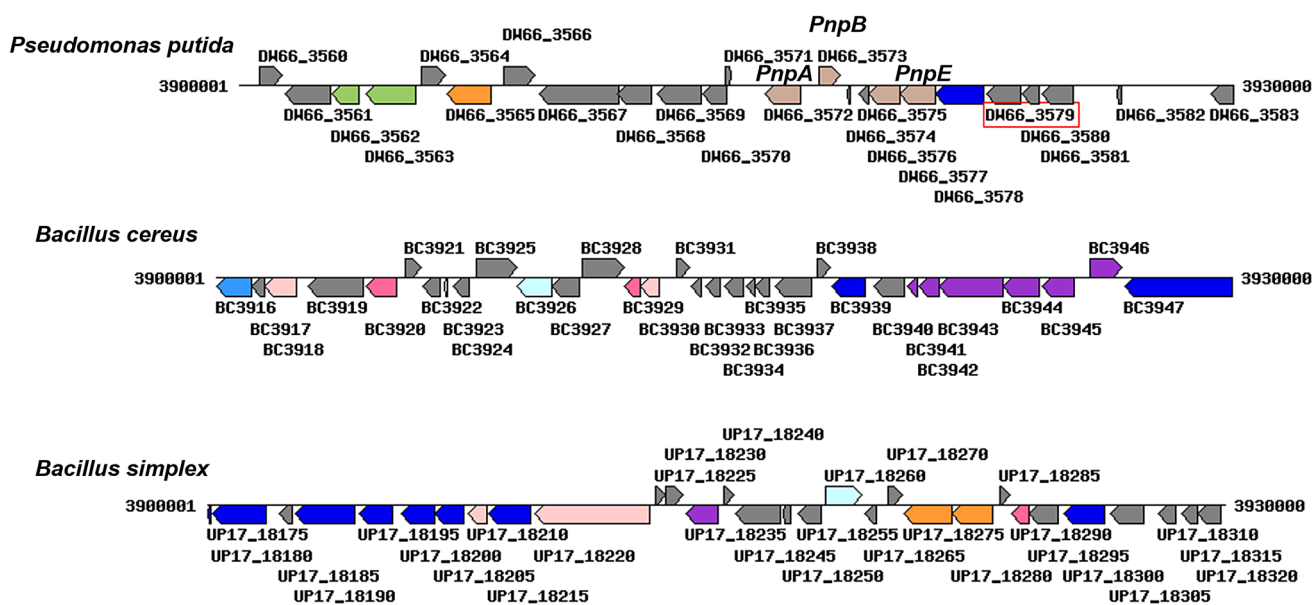


the phylogenetic tree, isolate 9 showed to be closely related to its species' type strain *Enterococcus faecium*<sup>T</sup> (Fig. 1).

The bacteria identified in the present work was already described as having an essential role in biodegradation processes due to their characteristics and adaptability.

When a high similarity of the obtained isolates with the type strains was detected, it was hypothesised that these strains might also degrade the drug. Thus, based on this assumption, it was searched if these strains have some genes known to encode for the enzymes with a particular role in paracetamol biodegradation. Although this information is relatively scarce in the literature, one of the most studied genomes is *Pseudomonas putida* DLL-E4, a Gram-negative bacterium isolated from a contaminated soil demonstrated to be able to use *p*-nitrophenol (PNP) as the sole carbon and energy source [35]. To our knowledge, it is still one of the most studied species in terms of genes that encode proteins (enzymes) related to PNP and hydroquinone (HQ)

degradation pathways. Thus, it can be inferred that these enzymes may play a role in paracetamol degradation since the PNP and HQ are metabolic products of this drug. Hu and colleagues [35] sequenced the whole genome of *Pseudomonas putida* DLL-E4 to characterise this species' genomic and metabolic diversity. Genes with predicted functions related to the catabolism of aromatic and heterocyclic compounds were found between bp 3,906,279 and 3,943,639 in the *Pseudomonas putida*'s genome and were designated as *pnp* degradation gene clusters containing *pnp* and *pnp1* genes. These genes are assumed to code for the following enzymes that may have been involved in the metabolism of PNP and HQ: ppud: DW66\_3572, K21725, 4-nitrocatechol/4-nitrophenol 4-monooxygenase (GenBank) *PnpA*, ppud: DW66\_3573, K23528, *p*-benzoquinone reductase (NADPH) (GenBank) *PnpB* and ppud: DW66\_3577, K00217, maleylacetate reductase (GenBank) *PnpE*.



**Fig. 2** Shows the representation of gene clusters in bp 3,900,001 to 3,930,000 in the genomes of *Pseudomonas putida*, *Bacillus cereus* and *Bacillus simplex*. Legend: Light brown: xenobiotics biodegradation and metabolism; Dark blue: carbohydrates metabolism; Orange:

amino acid metabolism; Purple: energy metabolism; Pink: genetic information processing; Dark pink: biosynthesis of other secondary metabolites (Color figure online)

The genetic potential (genes encoding similar functions are inferred from sequence homology) of *Pseudomonas putida* can be exploited for the degradation of PNP and related compounds such as paracetamol. With this purpose, when it was possible, a comparison of gene clusters of the whole genome of *Pseudomonas putida* with each type of strain was established to verify if these strains contain those genes (homologous genes). The comparison was performed with KEGG: Kyoto Encyclopedia of Genes and Genomes using genome comparison tools (gene cluster search), in which the respective genome map and gene list entry were assessed. The comparison was established for *Bacillus cereus*, *Bacillus simplex* (Fig. 2) and *Bacillus tropicus* (data not shown). It was impossible to compare *Corynebacterium nuruki* and *Enterococcus faecium* since these two species have only partial 16S rRNA sequences in the databases.

Also, *Pseudomonas putida* was compared with *Pseudomonas aeruginosa* since some research groups such as, for example, Karaman et al. [18] and Abdullah et al. [19] found that this bacterium was able to use paracetamol as the sole carbon and energy source.

From the previous analysis, no match (hit) was found with these specific gene clusters in the type strains *Bacillus cereus* and *Bacillus simplex* when compared with *Pseudomonas putida* (Fig. 2). Moreover, nor for *Pseudomonas aeruginosa* was any correspondence with these gene clusters found.

The comparison of these gene clusters shows that they may contain some conserved genes that probably encode similar functions, but in this particular case (for the genes responsible for the metabolisation of paracetamol and its metabolites), that does not seem to occur.

It is important to note that the type strains were only used to affiliate the isolates to the closest related species more accurately. Thus, even after a high similarity detection with the type strain, the isolates can present some different characteristics or have suffered mutations or even express some genes under certain experimental conditions that made them able to use paracetamol as a carbon source.

As suggested by Siezen [36], some homologous genes are lacking in individual gene clusters because maybe either (a) such genes are placed elsewhere, (b) such genes are not necessary at all, or (c) the sequencing of the gene cluster is incomplete.

A lot remains to explore in terms of genes that encode catabolic enzymes since, in these genomes, there are many hypothetical and uncharacterised proteins with unknown functions. There is the necessity of further genomic studies to better understand the genes involved in the metabolism of paracetamol by obtained isolates.

**Table 2** The IC<sub>50</sub> and EC<sub>50</sub> estimated for isolates 1, 3, 4 and 9 at different concentrations of paracetamol (mg/L) after 48 h of the experiment

Bacterial isolate	IC <sub>50</sub> (mg/L)	R <sup>2</sup>	EC <sub>50</sub> (mg/L)	R <sup>2</sup>
Isolate 1- <i>Bacillus cereus</i> group	1774 (1767 to 1781)	1	1947 (1702 to 2172)	0.956
Isolate 3- <i>Brevibacterium</i> <i>frigorigerans</i>	2212	1	3750 (2972 to 4754)	0.9603
Isolate 4- <i>Corynebacterium</i> <i>nuruki</i>	1944 (1941 to 1947)	1	4139 (2523 to 8917)	0.835
Isolate 9- <i>Enterococcus</i> <i>faecium</i>	1881 (1479 to 2492)	1	903.6 (604.7 to 1320)	0.8829

### IC<sub>50</sub> and EC<sub>50</sub> of Bacterial Isolates in the Presence of Paracetamol

The paracetamol concentration that inhibits the growth of half of the inoculum of each bacterial isolate (IC<sub>50</sub>) was estimated through the viability of bacteria in the presence of different concentrations of paracetamol and (EC<sub>50</sub>) given by the concentration of paracetamol necessary to produce 50% of the maximal effect.

The IC<sub>50</sub> and EC<sub>50</sub> were estimated in cultures inoculated with isolate 1, 3, 4 and 9 in nutrient broth and at different concentrations of paracetamol after 48 h of experiment. The bacterial viability as a function of the logarithm of paracetamol concentration fits a sigmoidal curve for all bacterial isolates (Table 2).

The IC<sub>50</sub> of each bacterial isolate is above the experimental paracetamol concentrations used, with all the values obtained above 1000 mg/L of the drug. Thus, it is not expected that the experimental concentrations would affect the performance of bacteria. The IC<sub>50</sub> values were of 1774 mg/L, 2212 mg/L, 1944 mg/L and 1881 mg/L of paracetamol for isolates 1, 3, 4 and 9, respectively (Table 2). Isolate 9 showed a different behaviour from the other isolates, with a gradual inhibition as concentrations increased, while all the other isolates were not inhibited at the lowest concentrations, starting to decrease after 1000 mg/L of the drug.

In a previous study with bacterial consortia, the IC<sub>50</sub> values obtained were 6.20 and 6.162 g/L for anaerobic and aerobic conditions, respectively [12]. However, it makes sense that isolates present a lower IC<sub>50</sub> since bacteria in a community can handle and withstand toxic conditions [12]. The EC<sub>50</sub> was estimated at 1947, 3750, 4139, and 903.6 mg/L. Again, isolate 9 revealed a different behaviour needing a

lower paracetamol concentration than the other isolates to produce 50% of the maximal effect (Table 2).

### Paracetamol Degradation by Bacterial Isolates

#### Paracetamol Degradation by Bacterial Isolates in Liquid Cultures

An independent experiment was performed to verify if these bacterial isolates used EDTA as a carbon or energy source. EDTA is a complexing agent and is used in the MSM medium in order to avoid precipitation of, e.g. Ca<sup>2+</sup>, Mg<sup>2+</sup> and PO<sub>4</sub><sup>3-</sup>, which in the absence of EDTA would form insoluble precipitates and while in the presence of this compound forms a soluble Ca(EDTA) complex, even in the presence of phosphate [37]. EDTA revealed antibacterial activity that is reduced by metal chelation of the ligand. Hennecken et al. [38] described a complete inhibition of a bacterial consortium by free EDTA, and the ability to degrade this compound depends on if it is complexed with equimolar quantities of calcium or magnesium ions. EDTA resistance to bacterial biodegradation is widely documented [e.g. 37, 38]. In an independent assay, it was verified that these bacteria could not use the EDTA, as a unique carbon source, in the quantity/conditions present in the MSM medium, which is consistent with the literature. Another aspect is that, so far, the identified bacterial strains with EDTA degrading abilities are all aerobic, gram-negative bacteria, whereas all the isolates are gram-positive [37].

In a previous preliminary test, it was verified that the maximum activity seemed to occur at around 48 h and that after this time, there were no significant changes in paracetamol degradation until 144 h, thus why this is the time interval considered in the present study.

**Table 3** Bacterial growth obtained by OD at 600 nm in cultures with MSM + paracetamol during the biodegradation assay. Values are expressed as the mean ± standard deviation (n = 3)

Incubation time (h)	Isolate 1 <i>Bacillus cereus</i> group	Isolate 3 [ <i>Brevibacterium</i> ] <i>frigorigerans</i>	Isolate 4 <i>Corynebacterium</i> <i>nuruki</i>	Isolate 9 <i>Enterococcus faecium</i>
48	0.118 ± 0.02	0.215 ± 0.004	0.079 ± 0.007	0.095 ± 0.004
96	0.204 ± 0.02*	0.423 ± 0.05	0.126 ± 0.007*	0.263 ± 0.04
144	*	0.437 ± 0.04	**	0.290 ± 0.003

\*OD affected (a clear or almost clear medium with bacterial clumps)

\*\*A high OD due to the appearance of a brown colour in the medium



Because the bacterial growth values are obtained in terms of culture's turbidity given by optical density measurements, in the cultures inoculated with isolate 1, *Bacillus cereus* group, and isolate 4 *Corynebacterium nuruki*, it was difficult to correctly measure it since both bacteria form clusters (aggregates). These float in the medium and produce glass adherent biofilm in the presence of glucose and paracetamol (Fig. 1S available in the ESM) that may affect OD measurements. This bacterial flocking/clumping can occur as a natural way for species to grow, but this can also be a mechanism to avoid media toxicity [39] or protection. For example, in some anaerobic species (usually facultative species, not strict anaerobes, as is the case of the *Bacillus cereus* group members), that behaviour allows them to grow in aerobic conditions.

The growth exhibited by [*Brevibacterium*] *frigorigerans* and *Enterococcus faecium* at 200 mg/L were low, as shown in Table 3.

Cultures performed in MSM + glucose as positive control presented a similar profile on the bacterial growth. However, in cultures of [*Brevibacterium*] *frigorigerans* and *Enterococcus faecium* in nutrient broth without drug, their growth was  $0.95 \pm 0.09$  and  $0.76 \pm 0.08$ , respectively, after 144 h incubation.

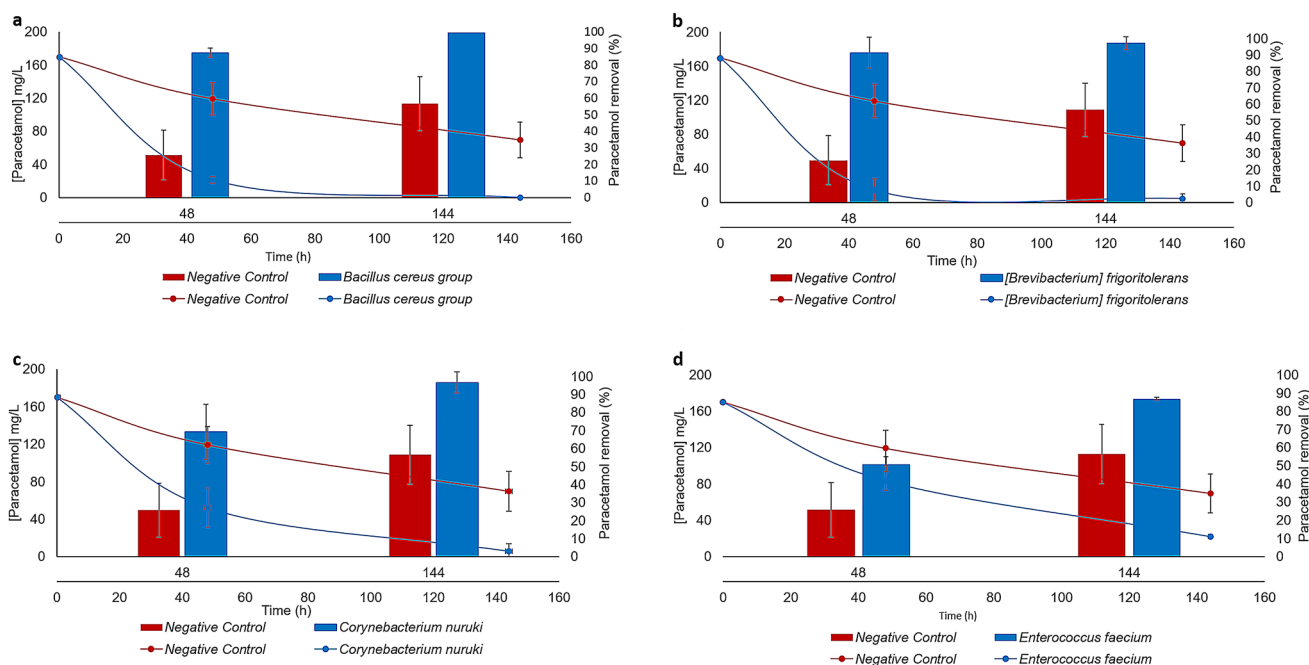
The slow bacterial growth in the presence of paracetamol can be explained in different ways. First as an adaptation of bacteria, i.e. aiming to gain resistance and to start

to use the drug only as a carbon source, since it was possible to detect drug removal during the time of the experiment, indicating that these bacteria may use paracetamol as a readily degradable carbon source, or bacteria which could co-metabolically degrade the drug as described for other drugs [40]. Secondly, as an accumulation of paracetamol within the bacterial cells (bioaccumulation) rather than adsorption at the cell surface (biosorption), as this last possibility also exists. For example, Moreira et al. [41] performed experiments where another drug, 2  $\mu\text{M}$  of fluoxetine, was used as the sole carbon source by *Labrys portucalensis* strain F11 in 30 days. They also reported that bacterium growth was about 0.25 after seven days of experiments, even when FLX was supplemented with acetate [41].

The obtained results of paracetamol removal are shown in Fig. 3.

When members of the species *Bacillus cereus* group, [*Brevibacterium*] *frigorigerans* and *Corynebacterium nuruki* were inoculated at 200 mg/L, a significant decrease in the concentration of paracetamol was observed, and a high percentage of drug removal was achieved (Fig. 3).

In the presence of *Bacillus cereus* group, [*Brevibacterium*] *frigorigerans*, *Corynebacterium nuruki* and *Enterococcus faecium*, removal of about  $87 \pm 3\%$ ,  $91 \pm 9\%$ ,  $69 \pm 15\%$  and  $51 \pm 4\%$  of 200 mg/L of paracetamol was obtained, respectively, after 48 h. In cultures with



**Fig. 3** Paracetamol degradation profile and removal (%) in MSM liquid cultures in the presence of 200 mg/L of drug inoculated with each one of the four bacterial isolates **a** *Bacillus cereus* group **b** [*Brevibacterium*] *frigorigerans* **c** *Corynebacterium nuruki* **d** *Enterococcus*

*faecium*, after 48 h and 144 h at 28 °C in dark conditions ( $n=3$ ; mean  $\pm$  standard deviation). N.D.=Not Detected, below the limit of detection

[*Brevibacterium*] *frigoritolerans*, *Corynebacterium nuruki* and *Enterococcus faecium*, the removal was about  $97 \pm 4\%$ ,  $97 \pm 6\%$  and  $86.9 \pm 0.8\%$  of 200 mg/L of the drug, respectively, after 144 h incubation (Fig. 3b, c, d). On the other hand, a species member of *Bacillus cereus* group apparently completely degraded 200 mg/L of paracetamol (Fig. 3a), without giving rise to toxic metabolites, since the peaks corresponding to 4-aminophenol, hydroquinone or benzoquinone were not detected during the chromatographic analyses, after 144 h incubation (Fig. 2S and 3Sb available in the ESM). On the other hand, cultures spiked with 200 mg/L of paracetamol without bacteria (negative control) presented a drug removal of  $26 \pm 15\%$  and  $56 \pm 16\%$  after 48 h and 144 h assay, respectively. If it is assumed that the physicochemical changes had the same participation in the abiotic and biotic systems, thus the biodegradation process performed by *Bacillus cereus* group, [*Brevibacterium*] *frigoritolerans*, *Corynebacterium nuruki* and *Enterococcus faecium* only contributed with 62%, 66%, 43.5% and 25% after 48 h of the assay, while after 144 h biotic contribution was apparently of about 40% for all isolates. However, when compared with the negative control *Bacillus cereus* group species, [*Brevibacterium*] *frigoritolerans*, *Corynebacterium nuruki* and *Enterococcus faecium* caused a significant decrease ( $P < 0.05$ ) in 200 mg/L of paracetamol, after 48 and 144 h incubation (Fig. 3).

In the negative control (without bacteria), the high percentages of paracetamol degradation after 144 h of assay can be due to several conditions and factors that may affect the stability of paracetamol, such as the ones described below.

Paracetamol is known to be unstable under humid conditions that can lead to its hydrolysis. Also, the rate of degradation of paracetamol in aqueous solutions is enhanced by temperature increase, exposure to light and oxygen, changes in solution's pH outside the range of 5–6 and by contamination with traces of 4-aminophenol [42–44]. Dietlin and Fredj [43] reported that a paracetamol solution in water at 50 °C suffered degradation after two weeks.

The negative controls were submitted to the same incubation conditions as the assays with bacteria, under agitation at 28 °C, which favoured the oxygenation of cultures. Thus, the oxygen that was not used by bacteria became available to react with paracetamol, leading to the oxidation and subsequent hydrolysis of the drug, speeding up its degradation. Khamis and colleagues [45] also found that the drug suffers from hydrolysis to 4-aminophenol when kept standing for some time (7 days) in wastewater sludge.

Although these specific bacterial isolates have not yet been described in the literature regarding their role in paracetamol biodegradation, for some of them, several studies have been conducted in what concerns their biodegrading activity on other organic compounds.

As mentioned, paracetamol biodegradation by a bacterial consortium was already tested by the group [12]. Thus the main aim of this work was to emphasise the importance of the isolates in the paracetamol degradation.

### Possible Degradation Pathway of Paracetamol by the Bacterial Isolates

The main aim of the present work was to search for bacterial isolates already existing in the environment, with the ability to biodegrade/remove paracetamol. Only a few organisms were reported to degrade this drug entirely. In spite this investigation did not intend to be a mechanistic study, it was decided to present the results of a preliminary assay aiming to eventually detect the compounds already known as metabolites of paracetamol based on the literature [e.g. 16–18] to be generated during the degradation of paracetamol by the obtained bacterial isolates and which can be putative metabolites. Microorganisms have developed successful approaches comprising specific enzymatic systems and metabolic pathways to access paracetamol as a carbon and energy supply [46].

In the present study, for cultures spiked with 200 mg/L of paracetamol incubated with *Bacillus cereus* group, *Corynebacterium nuruki* and *Enterococcus faecium* peaks that can match to hydroquinone (5.52 min) were detected. In contrast, for the isolate [*Brevibacterium*] *frigoritolerans* peaks matching with 4-aminophenol (4.57 min) and hexenoic acid (12.53 min) appeared after an incubation period of 48 h (Fig. 3.1S, ESM).

A species member of the *Bacillus cereus* group completely degraded the drug (mineralisation) since no peak was detected, whereas, in cultures of isolate [*Brevibacterium*] *frigoritolerans*, 2.8% of paracetamol remained to degrade, nevertheless no other important compounds were detected after 144 h incubation. In *Corynebacterium nuruki*, 3.4% of paracetamol and residual concentrations of hydroquinone were detected, whereas, for *Enterococcus faecium*, a higher percentage of paracetamol (13.1%) remained to degrade (Fig. 3.2S, ESM), after 144 h.

During the abiotic experiment, it was possible to observe a residual peak compatible with the 4-aminophenol and hydroquinone during the experiment (data not shown). The appearance of these compounds in the absence of bacteria could be due to paracetamol hydrolysis that is accelerated by the presence of oxygen, as above explained.

The culture with *Corynebacterium nuruki* presented a brown colour that may be correlated with the secondary metabolite 4-aminophenol, the main responsible for this medium colouration. Following the chromatogram (Fig. 3S, ESM), where a residual concentration of 4-aminophenol at 5.68 min, after 48 and 144 h of the assay was detected.

An examination of the results may predict that these isolates' mechanism of paracetamol degradation occurs in the same way as already described in other studies. Thus, paracetamol (C<sub>8</sub>H<sub>9</sub>NO<sub>2</sub>) may be initially catalysed by an amidohydrolase occurring in the cleavage of the bond between nitrogen and carbon from the carbonyl group with subsequent liberation of acetate to produce 4-aminophenol (C<sub>6</sub>H<sub>7</sub>NO), in which the amino group (nitrogen elimination) is subsequently substituted by a hydroxyl group (hydroxylation) to originate hydroquinone or 1,4-benzenediol (C<sub>6</sub>H<sub>6</sub>O<sub>2</sub>), after ring fission [14, 17, 47]. Zhang et al. [17] reported that in second place, paracetamol could initially suffer a hydroxylation, possibly catalysed by a hydrolytic enzyme, yielding hydroquinone (C<sub>6</sub>H<sub>6</sub>O<sub>2</sub>), a transitory metabolite, as it could be consequently oxidised to 1, 4-Benzoquinone (C<sub>6</sub>H<sub>4</sub>O<sub>2</sub>) that is more stable and believed to be a hazardous metabolite of benzene inducing genotoxic and mutagenic effects [16, 48].

Finally, Li, Ye and Gan [47] proposed that the reasonably high concentration of 2-hexenoic acids (C<sub>6</sub>H<sub>10</sub>O<sub>2</sub>) at 13.58 min, found in the samples, suggested the cleavage of the benzene ring, which may have contributed to subsequent paracetamol mineralisation.

As far as we know, our study is the first to demonstrate the paracetamol-degrading ability by a species member of *Bacillus cereus* group, [*Brevibacterium*] *frigoritolerans*, *Corynebacterium nuruki* and *Enterococcus faecium*.

## Conclusions

In the present study, bacteria with the ability to degrade paracetamol were isolated from aerobic sludge from an oxidation ditch's WWTP and identified utilising the 16S rRNA bacterial gene. This study indicates for the first time to our knowledge that members assigned to the *Bacillus cereus* group, [*Brevibacterium*] *frigoritolerans*, *Corynebacterium nuruki* and *Enterococcus faecium* were able to biodegrade paracetamol. Experiments using these isolates suggested that a *Bacillus cereus* group species, [*Brevibacterium*] *frigoritolerans*, *Corynebacterium nuruki* were apparently able to use 200 mg/L of paracetamol as a unique carbon source. A species from the *Bacillus cereus* group degraded 200 mg/L of paracetamol below the detection limit after 144 h of incubation. In both concentrations, the degradation occurred apparently without giving rise to toxic secondary metabolites after 144 h of incubation at 28 °C. *Enterococcus faecium* caused a significant decrease at 200 mg/L of paracetamol.

During the degradation process, the metabolites 4-aminophenol, hydroquinone and 2-hexenoic acid were detected as metabolic degradation intermediates of paracetamol. The obtained results pointed to a complete degradation

(mineralisation) of 200 mg/L paracetamol by a species member of the *Bacillus cereus* group, which can be promising, considering bioaugmentation strategies.

To the best of our knowledge, these species are reported for the first time as paracetamol degraders.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00284-021-02543-4>.

**Authors' Contributions** TP carried out the experimental work and wrote the article as part of her PhD. GM collaborated on part of the experimental work. Prof. MCC supervised the experimental work, corrected, and revised the manuscript. All authors read and approved the final manuscript.

**Funding** The authors would like to thank Fundação para a Ciência e a Tecnologia (FCT) for funding this research through the PhD grant SFRH/BD/95075/2013 and through the Centro de Ciências do Mar's Plurianual (UIDB/04326/2020). Financial support was obtained through project 0483\_PROBIOMA\_5\_E, co-financed by the European Regional Development Fund within the framework of the Interreg V-A Spain-Portugal program (POCTEP) 2014–2020.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

- Xu JJ, Hendriks BS, Zhao J, Graaf D (2008) Multiple effects of acetaminophen and p38 inhibitors: towards pathway toxicology. *FEBS Lett* 582:1276–1282. <https://doi.org/10.1016/j.febslet.2008.01.063>
- Vymazal J, Březinová TD, Koželuh M, Kule L (2017) Occurrence and removal of pharmaceuticals in four full-scale constructed wetlands in the Czech Republic—the first year of monitoring. *Ecol Eng* 98:354–364. <https://doi.org/10.1016/j.ecoleng.2016.08.010>
- Joss A, Zabczynski S, Göbel A, Hoffmann B, Löffler D, McArdell CS, Ternes TA, Thomsen A, Siegrist H (2006) Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. *Water Res* 40(8):1686–1696. <https://doi.org/10.1016/j.watres.2006.02.014>
- Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ (2009) The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. *Water Res* 43:363–380. <https://doi.org/10.1016/j.watres.2008.10.047>
- Papageorgiou M, Kosma C, Lambropoulou D (2016) Seasonal occurrence, removal, mass loading and environmental risk assessment of 55 pharmaceuticals and personal care products in a municipal wastewater treatment plant in Central Greece. *Sci Total Environ* 543:547–569. <https://doi.org/10.1016/j.scitotenv.2015.11.047>
- Pereira AMPT, Silva LJG, Lino CM, Meisel LM, Pena A (2016) Assessing environmental risk of pharmaceuticals in Portugal: an approach for the selection of the Portuguese monitoring stations in line with directive 2013/39/EU. *Chemosphere* 144:2507–2515. <https://doi.org/10.1016/j.chemosphere.2015.10.100>

7. Roberts PH, Thomas KV (2006) The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. *Sci Total Environ* 356:143–153. <https://doi.org/10.1016/j.scitotenv.2005.04.031>
8. Wilkinson J, Hooda PS, Barker J, Barton S, Swinden J (2017) Occurrence, fate and transformation of emerging contaminants in water: an overarching review of the field. *Environ Pollut* 231(1):954–970. <https://doi.org/10.1016/j.envpol.2017.08.032>
9. Jallouli N, Elghniji K, Trabelsi H, Ksibi M (2017) Photocatalytic degradation of paracetamol on TiO<sub>2</sub> nanoparticles and TiO<sub>2</sub>/cellulosic fiber under UV and sunlight irradiation. *Arab J Chem* 10(2):S3640–S3645. <https://doi.org/10.1016/j.arabjc.2014.03.014>
10. Scott PD, Bartkow M, Blockwell SJ, Coleman HM, Khan SJ, Lim R, McDonald JA, Nice H, Nugegoda D, Pettigrove V, Tremblay LA, Warne MS, Leusch FD (2014) A national survey of trace organic contaminants in Australian rivers. *J Environ Qual* 43(5):1702–1712. <https://doi.org/10.2134/jeq2014.01.0012>
11. Brumovský M, Bečanová J, Kohoutek J, Borghini M, Nizzetto L (2017) Contaminants of emerging concern in the open sea waters of the western Mediterranean. *Environ Pollut* 229:976–983. <https://doi.org/10.1016/j.envpol.2017.07.082>
12. Palma TL, Donaldben MN, Costa MC, Carlier JD (2018) Putative role of *Flavobacterium*, *Dokdonella* and *Methylophilus* strains in paracetamol biodegradation. *Water Air Soil Pollut* 229:200. <https://doi.org/10.1007/s11270-018-3858-2>
13. Peake BM, Braund R, Tong A, Louis A (2015) The life cycle of pharmaceuticals in the environment. In: *Biomedicine*. Woodhead Publishing Series, Cambridge, pp 110–136
14. Wu S, Zhang L, Chen J (2012) Paracetamol in the environment and its degradation by microorganisms. *Appl Microbiol Biotechnol* 96(4):875–884. <https://doi.org/10.1007/s00253-012-4414-4>
15. Marchlewicz A, Guzik U, Hupert-Kocurek K et al (2017) Toxicity and biodegradation of ibuprofen by *Bacillus thuringiensis* B1(2015b). *Environ Sci Pollut Res* 24(8):7572–7584. <https://doi.org/10.1007/s11356-017-8372-3>
16. De Gussem B, Vanhaecke L, Verstraete W, Boon N (2011) Degradation of acetaminophen by *Delftia tsuruhatensis* and *Pseudomonas aeruginosa* in a membrane bioreactor. *Water Res* 45:1829–1837. <https://doi.org/10.1016/j.watres.2010.11.040>
17. Zhang L, Hu J, Zhu R, Zhou Q, Chen J (2013) Degradation of paracetamol by pure bacterial cultures and their microbial consortium. *Appl Microbiol Biotechnol* 97(8):3687–4369. <https://doi.org/10.1007/s00253-012-4170-5>
18. Karaman R, Khamis M, Abbadi J, Amro A, Qurie M, Ayyad I, Ayyash F, Hamarshah O, Yaqmour R, Nir S, Bufo SA, Scranio L, Lerman S, Gur-Reznik S, Dosoretz CG (2016) Paracetamol biodegradation by activated sludge and photocatalysis and its removal by a micelle–clay complex, activated charcoal, and reverse osmosis membranes. *Environ Technol* 37(19):2414–2427. <https://doi.org/10.1080/09593330.2016.1150355>
19. Abdullah QY, Edrees WH, AL-Kaf AG, Najji KM (2018) Biodegradation of paracetamol by native bacterial strains isolated from Yemeni pharmaceutical wastewater plant in Sana'a. *Chron Pharm Sci* 2:512–522
20. Çeçen F, Gül G (2021) Biodegradation of five pharmaceuticals: estimation by predictive Models and comparison with activated sludge data. *Int J Environ Sci Technol* 18:327–340. <https://doi.org/10.1007/s13762-020-02820-y>
21. Lebaron P, Ghiglione J-F, Fajon C, Batailler N, Normand P (1998) Phenotypic and genetic diversity within a colony morphotype. *FEMS Microbiol Lett* 160:137–143. <https://doi.org/10.1111/j.1574-6968.1998.tb12903.x>
22. Fleury V, Gouyet J-F, Leonetti M (2001) Branching in Nature. Dynamics and morphogenesis of branching structures, from cell to river networks. Centre de Physique des Houches. Springer-Verlag, Berlin, Heidelberg
23. Palma TL, Shylova A, Costa MC (2021) Isolation and characterization of bacteria from activated sludge capable of degrading 17 $\alpha$ -ethinylestradiol, a contaminant of high environmental concern. *Microbiology*. <https://doi.org/10.1099/mic.0.001038>
24. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 35:1547–1549. <https://doi.org/10.1093/molbev/msy096>
25. Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 10:512–526. <https://doi.org/10.1093/oxfordjournals.molbev.a040023>
26. Kobori H, Sullivan CW, Shizuya H (1984) Bacterial plasmids in Antarctic natural microbial assemblages. *Appl Environ Microbiol* 48(3):515–518
27. Shimizu K (2013) Regulation systems of bacteria such as *Escherichia coli* in response to nutrient limitation and environmental stresses. *Metabolites* 4(1):1–35. <https://doi.org/10.3390/metab04010001>
28. Robador A, LaRowe DE, Finkel SE, Amend J, Nealson KH (2018) Changes in microbial energy metabolism measured by nanocalorimetry during growth phase transitions. *Front Microbiol* 19:109. <https://doi.org/10.3389/fmicb.2018.00109>
29. Logan NA (2010) *Bacillus anthracis*, *Bacillus cereus*, and Other Aerobic endospore-forming bacteria. In: Boriello SP, Murray PR, Funke G (eds) *Topley and Wilson's Microbiology and Microbial Infections*. Wiley, Hoboken, pp 922–952
30. Liu Y, Du J, Lai Q, Zeng R, Ye D, Xu J, Shao Z (2017) Proposal of nine novel species of the *Bacillus cereus* group. *Int J Syst Evol Microbiol* 67:2499–2508. <https://doi.org/10.1099/ijsem.0.001821>
31. Miller RA, Beno SM, Kent DJ, Carroll LM, Martin NH, Boor KJ, Kovac J (2016) *Bacillus wiedmannii* sp. nov. a psychrotolerant and cytotoxic *Bacillus cereus* group species isolated from dairy foods and dairy environments. *Int J Syst Evol Microbiol* 66(11):4744–4753. <https://doi.org/10.1099/ijsem.0.001421>
32. Liu Y, Lai Q, Göker M, Meier-Kolthoff JP, Wang M, Sun Y, Wang L, Shao Z (2015) Genomic insights into the taxonomic status of the *Bacillus cereus* group. *Sci Rep* 16(5):14082. <https://doi.org/10.1038/srep14082>
33. Gelsomino R, Vancanneyt M, Vandekerckhove TM, Swings J (2004) Development of a 16S rRNA primer for the detection of *Brevibacterium* spp. *Lett Appl Microbiol* 38:532–535. <https://doi.org/10.1111/j.1472-765X.2004.01533.x>
34. Beesley CA, Vanner CL, Helsel LO, Gee JE, Hoffmaster AR (2010) Identification and characterization of clinical *Bacillus* spp. isolates phenotypically similar to *Bacillus anthracis*. *FEMS Microbiol Lett* 313(1):47–53. <https://doi.org/10.1111/j.1574-6968.2010.02120.x>
35. Hu X, Wang J, Wang F, Chen Q, Huang Y, Zhongli C (2014) Complete genome sequence of the p-nitrophenol-degrading bacterium *Pseudomonas putida* DLL-E4. *Genome A* 2(3):e00596-e614. <https://doi.org/10.1128/genomeA.00596-14>
36. Siezen RJ, Kuipers OP, de Vos WM (1996) Comparison of lantibiotic gene clusters and encoded proteins. *Antonie Van Leeuwenhoek* 69(2):171–184. <https://doi.org/10.1007/BF00399422>
37. Rodríguez J, Oviedo C (2003) EDTA: the chelating agent under environmental scrutiny. *Quim Nova* 26(6):901–905. <https://doi.org/10.1590/S0100-40422003000600020>
38. Henneken L, Nörtemann B, Hempel DC (1995) Influence of physiological conditions on EDTA degradation. *Appl Microbiol Biotechnol* 44:190–197. <https://doi.org/10.1007/BF00164501>
39. Crosby HA, Kwiecinski J, Horswill AR (2016) *Staphylococcus aureus* aggregation and coagulation mechanisms, and their function in host-pathogen interactions. *Advances in Applied*

- Microbiology. Elsevier, Amsterdam, pp 1–41. <https://doi.org/10.1016/bs.aams.2016.07.018>
40. Fioravante IA, Albergaria B, Teodoro TS, Magalhães SMS, Barbosa F, Augusti R (2012) Removal of 17 $\alpha$ -ethinylestradiol from a sterile WC medium by the cyanobacteria *Microcystis novacekii*. *J Environ Monit* 14(9):2362–2366. <https://doi.org/10.1039/c2em30320e>
  41. Moreira IS, Ribeiro AR, Afonso CM, Tiritan ME, Castro PML (2014) Enantioselective biodegradation of fluoxetine by the bacterial strain *Labrys portucalensis* F11. *Chemosphere* 111:103–111. <https://doi.org/10.1016/j.chemosphere.2014.03.022>
  42. IARC Monographs (2020) Paracetamol, 50:307–332. <https://monographs.iarc.fr/wp-content/uploads/2018/06/mono50-20.pdf>. Accessed 23 July 2020
  43. Dietlin F, Fredj D (2000) Stable liquid paracetamol compositions, and method for preparing same. United States Scr, Pharmatop (FR) 6028222. <http://www.freepatentsonline.com/6028222.html>. Accessed on 23 July 2020
  44. Chiam E, Weinberg L, Bellomo R (2015) Paracetamol: a review with specific focus on the haemodynamic effects of intravenous administration. *Heart Lung Vessel* 7(2):121–132
  45. Khamis M, Karaman R, Ayyash F, Qtait A, Deeb O, Manssra A (2011) Efficiency of advanced membrane wastewater treatment plant towards removal of aspirin, salicylic acid, paracetamol and *p*-Aminophenol. *Indian J Environ Health* 5:121–137
  46. Ahmed Z, Sarwar Z, Ahmad N, Hussain MS, Riazuddin S (1999) Genetic modification of *Pseudomonas putida* for enhanced degradation of phenol and nitrophenol. *Pak J Biochem Mol Biol* 32:32–37
  47. Li J, Ye Q, Gan J (2014) Degradation and transformation products of acetaminophen in soil. *Water Res* 49:44–52. <https://doi.org/10.1016/j.watres.2013.11.008>
  48. Westphal GA, Bünger J, Lichey N, Taeger D, Mönnich A, Hallier E (2009) The benzene metabolite para-benzoquinone is genotoxic in human, phorbol-12-acetate-13-myristate induced, peripheral blood mononuclear cells at low concentrations. *Arch Toxicol* 83(7):721–729. <https://doi.org/10.1007/s00204-009-0402-6>

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