

Toxicity of Diclofenac and its Biotransformation by *Raoultella* sp. DD4

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Abstract

In recent years the increased consumption of diclofenac, a biologically active compound that is toxic to organisms and persistent to biodegradation, has resulted in its presence in the environment. This is the first report on the biotransformation of diclofenac by a pure bacterial strain, *Raoultella* sp. DD4, which is able to transform 0.6 mg/L of diclofenac in 28 days. Additionally, strain DD4 is more resistant to diclofenac than other tested organisms. The estimated value for EC50 for this strain is 1.95 g/L. This is approximately five-fold higher than the value of microbial toxic concentration MTC_{avg} (0.416 g/L). Moreover, genotoxicity studies have indicated that diclofenac is not a mutagenic compound.

Keywords: biotransformation, diclofenac, toxicity, *Raoultella*

Introduction

The global consumption of non-steroidal anti-inflammatory drugs has dramatically increased in recent years. Although such drugs can improve quality of life, they also contribute to increasing environmental pollution. Among the non-steroidal anti-inflammatory drugs, diclofenac has been added to a list of 33 pollutants that are monitored and controlled in European Union surface water [1-2]. Diclofenac is not completely metabolized in organisms and it is discharged as a contaminant to wastewater treatment plants. Because most wastewater treatment plants are unable to remove diclofenac, it has been detected in groundwater, surface water, and wastewater effluents, as well as in drinking water at concentrations ranging between ng/L and µg/L [2-4].

Although concentrations of diclofenac in the environment do not cause acute toxicity, chronic toxicity is potentially possible. For example, in some studies histopathological effects on the liver, kidney, and gills of trout at concentrations of 1.0-5.0 µg/L have been observed [2-3]. Additionally, the high octanol-water partition coefficient of diclofenac may cause bioaccumulation of this drug in fish or other organisms, and may be a subsequent potential secondary poison in the food chain [3].

Diclofenac undergoes decomposition by physico-chemical factors and it is generally resistant to biological treatment [5]. However, physico-chemical transformation leads to the formation of potentially harmful by-products while the biological techniques are safer and more cost-effective. Unfortunately, little is known about microorganisms able to degrade or biotransform diclofenac. *Trametes versicolor* and the white rot fungi *Phanerochaete chrysosporium* and *Phanerochaete sordida* were described as being able to biotransform diclofenac.

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During these processes hydroxylated derivatives were found. This suggests that hydroxylating enzymes such as laccase or cytochrome P-450 are probably engaged in diclofenac biotransformation [6-9]. Bacteria able to degrade this compound are still unknown.

In light of the above-mentioned facts it is very important to study the microbiological degradation of diclofenac and its impact on microorganisms. Therefore, the aim of our work was to investigate the toxicity of diclofenac to different microorganisms, and the ability of *Raoultella* sp. DD4 to degrade diclofenac.

Materials and Methods

Phylogenetic Analysis

Bacterial DNA was isolated from pure culture using the DNA commercial kit (GenElute Bacterial Genomic DNA Kit, Sigma-Aldrich). 16S rRNA gene amplification, sequencing and sequence analysis were carried out as described by Guzik et al. [10]. The 16S rRNA gene sequence determined in this study has been deposited in the GeneBank database under accession number KU684448.

Morphological, Physiological, and Biochemical Characterization of the DD4 Strain

The DD4 strain was phenotypically and biochemically characterized using standard techniques (Gram staining, colony shape, size and colour on nutrient agar plate, etc.), according to *Bergey's Manual of Determinative Bacteriology* [11]. Additional biochemical and physiological characteristics were determined using the API 20NE system (BioMerieux, Lyon, France).

Diclofenac Degradation Experiment

Raoultella sp. DD4 was routinely cultivated in the nutrient broth at 30°C and 130 rpm for 24 hours. After this, cells were harvested by centrifugation (5,000 x g at 4°C for 15 min), washed with a fresh sterile medium, and used as inoculum.

Degradation of diclofenac was performed in 500 ml Erlenmeyer flasks containing 250 ml of a mineral salt medium [12] and inoculated with cells to a final optical density of about 1.0 at $\lambda = 600$ nm (OD₆₀₀). Diclofenac was added to obtain a final concentration of 6 mg/L, and all cultures were incubated with shaking at 30°C for 28 days. Chromatographic analyses of the culture fluid and measurements of culture growth were carried out every seven days. All cultures were grown in triplicate.

Additionally, two control cultures (250 mL) were prepared: an uninoculated control consisting only of the mineral salt medium (abiotic degradation control), and a heat-killed control consisting of bacterial cells destroyed by autoclaving (adsorption onto biomass control). The optical density of the heat-killed control was the same as for the examined cultures.

Determination of Substrate Concentration

The concentration of diclofenac was determined with the HPLC technique using the Merck Hitachi HPLC reversed-phase chromatograph equipped with an Ascentis Express C18 HPLC Column (100 x 4.6mm), an Opti-Solw EXP pre-column, and a UV/VIS DAD detector. The mobile phase composed of acetonitrile, 1% acetic acid, and methanol (50:30:20 v/v) at a flow rate of 1 mL/min was used during analysis of the diclofenac concentration. The detection wavelength was set at 276 nm. Diclofenac in supernatant was identified by comparing the HPLC retention time and UV-visible spectra with that of the external standard.

Bacterial Growth Inhibition Test

To determine the inhibitory effect of diclofenac on bacterial growth, a pure culture of *Raoultella* sp. DD4 was grown in a nutrient broth supplemented with diclofenac in a concentration range of 0-3.0 g/L. The initial optical density of each culture was 0.1 (OD₆₀₀). After 24 hours of incubation with shaking at 30°C, the optical density of the cultures was measured. The EC₅₀ value was estimated using GraphPad PRISM 6.05 software.

Toxicity Bioassays

We used the MARA test to assess the acute toxicity of diclofenac toward various microorganisms [13]. The MTC value was calculated according to the formula:

$$\text{MTC} = c_{\min} \times d \left(\frac{P_{\text{tot}}}{P_o} \right)^{-1} \quad (1)$$

...where c_{\min} is the lowest concentration in the gradient, P_o is pellet size in the control, d is dilution factor, and P_{tot} is sum of all the pellets sizes across the concentration gradient [13-15].

Ames MPF tests (Xenometrix) were performed with *Salmonella typhimurium* TA98 and *Salmonella typhimurium* TA100 according to the manufacturer's recommendations. Ten mL of the growth medium were inoculated with 10 μ l of refrozen bacterial strains and incubated for 16 hours at 37°C with shaking at 250 rpm in the presence of 50 μ g/mL ampicillin until culture density reached >2.0 (OD₆₀₀). Next, the cultures were diluted 10-fold in the exposure medium, and 240 μ L of the mixture was introduced into every well of the 24-well plate.

Simultaneously, an appropriate volume of 600 mg/L diclofenac stock solution was introduced into the wells to obtain the final concentrations of 18.75, 37.50, 75, 150, 300, and 600 mg/L. Diluted inocula of bacterial strains were negative controls. The positive controls comprised the diluted inoculum and 0.1 μ g/mL 4-nitroquinoline-N-oxide or 2.0 μ g/mL 2-nitrofluorene as mutagens. The mutagenicity assays were also conducted in the presence of metabolic activation fraction S9 (rat liver microsomal fraction). The samples were prepared as described above,

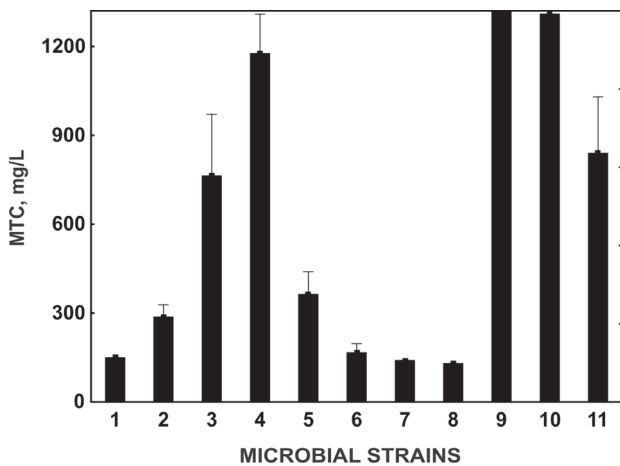


Fig. 1. Mean MTC values in the MARA test, with diclofenac exposure ranging between 180 and 1330 mg/L. Microorganisms used in the MARA test: 1 - *Microbacterium* sp. G(+); 2 - *Brevundimonas diminuta* G(-); 3 - *Citrobacter freundii* G(-); 4 - *Comamonas testosteroni* G(-); 5 - *Enterococcus casseliflavus* G(+); 6 - *Delftia acidovorans* G(-); 7 - *Kurthia gibsonii* G(+); 8 - *Staphylococcus warneri* G(+); 9 - *Pseudomonas aurantiaca* G(-); 10 - *Serratia rubidaea* G(-); and 11 - *Pichia anomala* (yeast).

except that the microsomal fraction was further introduced into each well. Diluted inocula of bacterial strains were negative controls. The positive controls consisted of diluted inocula of bacteria, fraction S9, and 0.5 or 1.25 µg/mL 2-aminoanthracene for strains TA98 or TA100, respectively.

The 24-well plates were then incubated for 90 minutes at 37°C with shaking at 250 rpm, and the cultures were mixed with the indicator medium. Then, 50 µL aliquots of each culture were replica plated into 48 wells of a 384-well plate and incubated at 37°C for 48 hours. The number of positive wells out of 48 wells per replicate and per tested concentration of ibuprofen was compared with the number of spontaneous revertants obtained in the negative control section [16].

Results and Discussion

Toxicity of Diclofenac

Diclofenac is a biologically active compound. Therefore, this drug and products of its transformation in vertebrates and in the environment may increase ecological risk [17]. Knowledge concerning the impact of diclofenac on bacteria is limited. The EC50 values estimated by Ferrari et al. [18] in the Microtox test with *Vibrio fischeri* as a standard microorganism amounted to 11.454 mg/L of diclofenac. For cyanobacteria *Synechococcus leopolensis* the non-observed-effect concentration for diclofenac was 10 mg/L, while the inhibition of growth of Gram-positive and Gram-negative bacteria was observed at 50 to 100 mg/L [19]. Moreover, diclofenac influences the

structural divergence of the bacterial population in active sludge and reduced bacterial diversity, leading to functional changes in active sludge wastewater treatment systems [20]. Because of this, it is especially important to investigate the effect of diclofenac on a pure bacteria strain able to degrade this drug.

To evaluate the acute toxicity of diclofenac in bacteria we used the MARA test, which was carried out with 10 bacterial strains and one yeast strain [13].

We observed different responses of the tested microorganisms. The most sensitive microorganism was Gram-positive *Staphylococcus warneri* (Fig. 1). The MTC for this strain was 130.00 ± 0.0 mg/L, whereas the most resistant strains were *Pseudomonas aurantiaca* and *Serratia rubidaea* (MTC above 1300 mg/L). The mean value of microbial toxic concentration MTC_{avg} for diclofenac, which is the equivalent of EC₅₀, was 416.67 ± 5.77 mg/L. According to EU Directive 93/67/EEC, substances with EC₅₀ > 100 mg/L are classified as nontoxic compounds to aquatic organisms [21-22].

We also examined the mutagenic potential of diclofenac. Table 1 presents a summary of the results of the Ames mutagenicity assay. In this test, two model organisms – *Salmonella typhimurium* TA98 and *Salmonella typhimurium* TA100 – were treated with diclofenac. We showed that diclofenac did not induce a significant dose-related increase in the number of revertant colonies in the tested strains either in the presence or absence of the metabolic activation system. According to U.S. Environmental Protection Agency and GenPharmTox guidelines (mutant frequency above two-fold greater than the control frequency obtained in the same experiment) indicates the potential mutagenic influence of the compound [23]. In our study, none of the results of the Ames test exceeded the critical value, 2.0.,

Table 1. Differential phenotypic characteristics of strain DD4.

Characteristic	Results	Characteristic	Results
Hydrolysis of esculin	+	Urease	-
Hydrolysis of gelatin	-	Indol production	-
Arginine dihydrolase	-	Nitrate reduction	+
Glucose fermentation	+		
Assimilation of:			
Glucose	+	Mannitol	+
Arabinose	+	Maltose	+
Mannose	+	Gluconate	+
Malate	-	Caprate	-
Citrate	-	Adipate	-
Phenylacetate	-	N-acetylglucosamine	+

+ positive reaction, - negative reaction

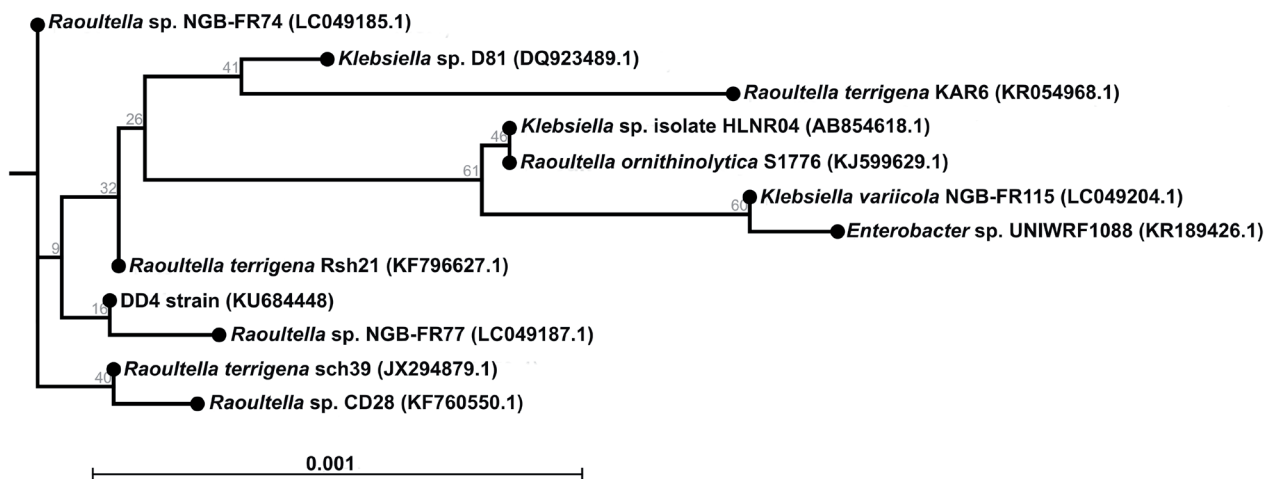


Fig. 2. Neighbour-joining tree showing the phylogenetic position of diclofenac-degrading bacterial strain DD4 and related species based on partial 16S rRNA gene sequences. The GenBank accession number for each microorganism used in the analysis is shown in parentheses after the species name. Bootstrap values (expressed as a percentage of 100 replications) are shown at the branch.

and no mutagenic activity of diclofenac in *Salmonella* strains TA98 and TA100 was observed. These results are in accordance with the results of Philipose et al. [24].

Although the lack of a mutagenic effect and weak toxic effect of diclofenac on microorganisms was observed,

the influence of diclofenac on processes in vertebrates is not excluded. For example, Chen et al. [2] showed that diclofenac altered the expression of genes important for the development of the cardiovascular and nervous systems in zebrafish.

Table 2. Mutagenic activity expressed as the mean and standard deviations of the number of revertants/plate in bacterial strains TA98 and TA100 treated with diclofenac, at various doses, without metabolic activation system or with metabolic activation system (S9).

Dose level (mg/L)	Without metabolic activation system					
	TA98			TA100		
	Number of revertants/plate	Fold increase (over baseline 2.15)	t-test p-value	Number of revertants/plate	Fold increase (over baseline 17.35)	t-test p-value
0	0.50±0.84	-	-	14.83±3.06	-	-
18.75	2.33±2.52	1.75	0.07	16.33±5.13	0.91	0.30
37.50	0.67±0.58	0.50	0.38	14.67±2.08	0.82	0.47
75	1.33±0.58	1.00	0.09	14.00±2.65	0.78	0.35
150	0.67±1.15	0.50	0.40	10.00±5.57	0.56	0.06
300	1.67±1.15	1.25	0.06	11.67±0.58	0.65	0.06
600	1.33±0.58	1.00	0.09	12.00±4.00	0.67	0.14
Dose level (mg/L)	With metabolic activation system					
	TA98			TA100		
	Number of revertants/plate	Fold increase (over baseline 3.00)	t-test p-value	Number of revertants/plate	Fold increase (over baseline 19.80)	t-test p-value
0	1.17±0.75	-	-	12.67±4.41	-	-
18.75	1.33±0.58	0.69	0.37	12.00±6.24	0.70	0.43
37.50	1.33±0.58	0.69	0.37	8.00±1.00	0.47	0.06
75	2.00±1.00	1.04	0.10	9.00±1.00	0.53	0.11
150	0.67±1.15	0.35	0.23	16.00±3.61	0.94	0.15
300	1.00±1.00	0.52	0.39	12.33±2.08	0.72	0.45
600	1.00±1.00	0.52	0.39	14.33±7.23	0.84	0.34

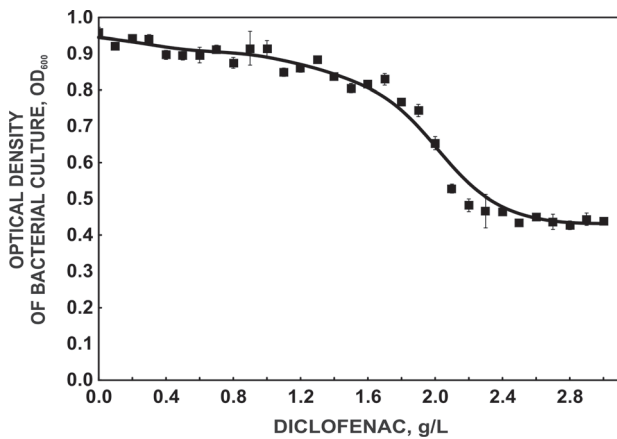


Fig. 3. Growth inhibition of *Raoultella* sp. DD4 in the presence of different diclofenac concentrations.

Biotransformation Studies

Until now, none of the bacterial strains have been isolated and characterized as diclofenac degraders.

The strain marked as DD4 is a Gram-negative rod-shaped bacterium. Colonies of this strain were found to be circular, smooth, glossy, and cream-colored. The biochemical and physiological characteristics of strain DD4 are summarized in Table 2. Partial sequence analysis of the 16S rRNA gene allows for the classification of the isolate with 100% similarity as a member of the genus *Raoultella* (Fig. 2). In accordance with these data, the isolate DD4 was classified in the genus *Raoultella* and named *Raoultella* sp. DD4.

It has been reported that diclofenac in concentrations between 50 and 100 mg/L may be toxic to bacteria [25]. The EC_{50} of diclofenac obtained using *Raoultella* sp. DD4 grown in the nutrient broth supplemented with diclofenac in the concentration range from 0.0 to 3.0 g/L was 1.95 g/L (Fig. 3). The estimated value for EC_{50} is approximately five-fold higher than the value of microbial

toxic concentration MTC_{avg} . This indicates that the DD4 strain is especially resistant to diclofenac.

The resistance of the DD4 strain to diclofenac allowed for the supposition that this strain may be a good candidate for the biotransformation and/or biodegradation of this drug. The measurements of diclofenac in the uninoculated control (I) as well as in the heat-killed control (II) after 28 days of incubation revealed no changes in the concentration of the compound. This confirmed the lack of adsorption of diclofenac to the cell surface and chemical oxidation of this drug. Therefore, the ability of strain DD4 to degrade 6 mg/L of diclofenac was examined. As shown in Fig. 4, approximately 10% (600 μ g/L) of diclofenac was removed over 28 days. The degradation of diclofenac was accompanied by a decrease in the number of bacteria. As the used concentration of diclofenac is non-toxic for *Raoultella* sp. DD4, we may suppose that this compound is not a sufficient carbon and energy source for strain DD4. Until now, no data have been available on pure bacterial strains able to degrade or transform diclofenac. Only the mixed microbial cultures, derived mainly from activated sludge, were examined with a focus on the possibility of diclofenac decomposition in the concentration range 296 μ g/L to 300 mg/L [4, 26]. The only strain that is able to utilize diclofenac with high efficiency in the pure culture is the fungus *Phanerochaete sordida* YK-624 [7].

Conclusions

Raoultella sp. DD4 is able to transform 0.6 mg/L of diclofenac over 28 days. However, diclofenac is not a sufficient carbon source for this strain. On the other hand, strain DD4 is uniquely resistant to diclofenac because the value of the EC_{50} for this strain is approximately five-fold higher than the value of the microbial toxic concentration MTC_{avg} . Toxicity studies indicate the lack of a mutagenic effect of diclofenac on microorganisms, but it does not exclude the possibility that diclofenac may influence organisms in the environment.

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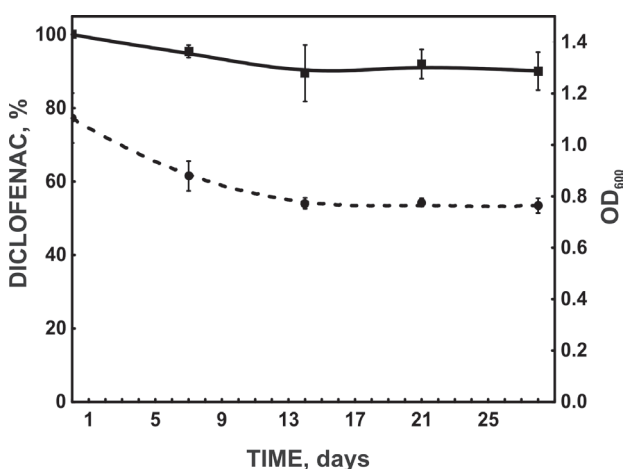


Fig. 4. Degradation of 6 mg/L diclofenac by *Raoultella* sp. DD4 and changes of microbial biomass monitored as optical density at 600 nm.

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